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THE UNIVERSITY OF ALBERTA

IDENTIFICATION AND PARTIAL PURIFICATION OF THE HUMAN ERYTHROCYTE RECEPTOR FOR CFA/I PILI

by

Peter Pieroni

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF
Master of Science

Department of Medical Microbiology and Infectious Diseases

EDMONTON, ALBERTA
Spring, 1988

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THE UNIVERSITY OF ALBERTA
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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled IDENTIFICATION AND PARTIAL PURIFICATION OF THE HUMAN ERYTHROCYTE RECEPTOR FOR CFA/I PILI submitted by Peter Pieroni in partial fulfilment of the requirements for the degree of Master of Science in Medical Microbiology and Infectious Diseases.

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ABSTRACT

Human erythrocyte receptors for CFA/I pili were investigated. Erythrocyte binding assays, using whole organisms and enzyme- treated (trypsin and neuraminidase) erythrocytes suggest that the CFA/I receptor is a glycoprotein with important sialic acid moieties. A soluble glycoprotein fraction was obtained from human erythrocyte membranes by extraction with lithium diiodosalicylate. The extract fraction caused agglutination of the CFA/I^+ but not the CFA/I^- organisms at a protein concentration of 0.5 mg/mL. A CFA/I pili receptor was identified in iodinated extracts by utilizing a binding assay using whole bacterial cells. Subsequent analysis of the iodinated, bacterial-bound material by SDS polyacrylamide gel electrophoresis and autoradiography revealed a polypeptide with an apparent molecular weight (MW) of 27,000 ± 1,500, which was present in the original labeled extract and on CFA/I^+ organisms but was not observed on extract-coated CFA/I^- bacteria. In addition, when purified CFA/I pili were added to the whole organism binding assay mixture, they reduced the amount of the 27,000 MW species which bound to CFA/I^+ bacteria. Wheat germ agglutinin (WGA) affinity chromatography of [^{125}I]- labeled extract revealed that the 27,000 MW band contains sialyloligosaccharide units. This observation is consistent with the results of erythrocyte binding experiments.

Scatchard analysis of equilibrium binding data obtained using CFA/I coated polystyrene test tubes and radioiodinated extract demonstrated that the interactions between CFA/I pili and their erythrocyte receptor were non-cooperative. Further, the receptor binding sites appeared to be homogeneous and binding is saturated. The observation that the [^{125}I]-labeled extract was able to bind to WGA, CFA/I$^+$ bacteria, CFA/I coated polystyrene test tubes, and the demonstration that purified CFA/I pili could compete for the [^{125}I]-labeled extract binding provided evidence that the binding activity of the receptor species were not altered by the iodination procedure.

The data also indicated that there are non-specific mechanisms by which both CFA/I$^+$ and CFA/I$^-$ strains of H10407 *E. coli* are able to adhere to the erythrocytes.

Electron microscopic investigation revealed that CFA/I pili binds to the erythrocyte membrane via the pilus tip, which is in agreement with published data.

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ABBREVIATIONS

MSHA	mannose sensitive hemagglutination
MRHA	mannose resistant hemagglutination
PBS	phosphate buffered saline
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
McAb	monoclonal Antibody
Mdal	megadaltons
Kdal	kilodaltons
PTA	phosphotungstic acid
BSA	bovine serum albumin
EDTA	disodium ethylene diamine tetraacetate
CFA/I	colonization factor antigen I
LPS	lipopolysaccharide
WGA	wheat germ agglutinin
TCA	trichloroacetic acid
Man	mannose
Glc NAc	N-acetyl glucosamine
NeuAc	N-acetyl neuraminic acid
Gal	galactose

1. INTRODUCTION

1.1 Diarrheagenic *Escherichia coli* :

The pathogenicity of certain *Escherichia coli* strains for humans is well documented in the literature (23, 26, 31, 32, 36, 40, 45, 48, 56, 63, 64, 65, 89, 93, 94, 95, 98, 106, 108, 109, 113). However when Theodore Escherich initially described *E. coli* in 1885, he was not convinced the organisms were pathogenic, because they were found to be universally present in the intestines of healthy individuals. However, in 1945, John Bray published an article on the association of antigenically homogenous *E. coli* with summer diarrhea (10). It was then that the bacterium was accepted as a human pathogen. Subsequent to Bray's study, a system for serogrouping *E. coli* was developed.

The system is based on serological reactions to antigens exposed on the surface of the bacteria. In *E. coli*, as in other gram negative bacteria, the outer membrane contains lipopolysaccharide (LPS) and protein in addition to phospholipids. The LPS is composed of the lipid A, core polysaccharide, and antigenically variable (group specific) oligosaccharide repeating units, termed the O antigens. To date over 168 serologically distinct O antigens have been described in *E. coli* (68). In addition to LPS, the flagella are responsible for production of type-specific antibodies (H antigens). The capsular or K antigens are generally complex polysaccharides, which surround the outer membrane of some strains of *E. coli*. Unfortunately, prior to extensive EM studies some pili types were mistaken for the K

antigens. Although the true nature of these antigens is now documented a consensus on how to modify the confusing nomenclature has not been reached.

In 1949, Giles et al. (45), and Taylor et al. (108) independently reported the results of investigations into outbreaks of diarrhea in two institutional centres (a nursery and a maternity hostel, respectively) and identified the etiological agent as a strain of *E. coli*. These investigations confirmed the results of Bray's earlier studies.

In 1954, Koya, et al. (64, 65) published a paper on the multiplication of *Escherichia coli* serotype O-111 in the intestinal tracts of adult volunteers. They utilized an intubation technique to demonstrate that *E. coli* O111 must multiply in the lower jejunum or upper ileum of the small intestine in order to effect diarrheal symptoms. This inevitably demonstrated that some strains of *E. coli* can be pathogenic to humans when displaced from their natural ecological niche in the large intestine to a region of the gastrointestinal tract that is relatively sterile in healthy individuals.

In addition to its enteric pathogenicity, *E. coli* has also emerged as an important opportunistic pathogen. *E. coli* is thus recognized as an important cause of extraintestinal infections such as: urinary tract infections, wound infections, peritonitis, meningitis, pneumonia due to gram negative bacteria, and septicemia (58, 63, 68, 85, 88).

To date, diarrheagenic *E. coli* can be placed into four categories based on: interactions with intestinal mucosa, clinical

symptoms of the disease, epidemiology and O:H serotypes. These categories are: (1) enteropathogenic *E. coli* (EPEC); (2) enterotoxigenic *E. coli* (ETEC); (3) enteroinvasive *E. coli* (EIEC); and (4) enterohemorrhagic *E. coli* (EHEC). There are other strains of diarrheagenic *E. coli*, which do not fall into any of these four particular categories. These have recently been assigned to a fifth group, termed "enteroadherent *E. coli* " (EAEC; 68), see Table 1.

1.2 Enterotoxigenic *Escherichia coli* :

The recognition of enterotoxigenic *E. coli*-mediated diarrhea in man was made in the early 1970's, largely based on the studies of Gorbach and co-workers in Calcutta (5,48). They examined adults exhibiting cholera-like symptoms but could not isolate *Vibrio cholerae* from stool specimens obtained from patients. Although the patients had symptoms indistinguishable from cholera (rice watery stool, nausea, abdominal cramps, low grade fever, profound dehydration and shock) the duration of diarrhea was relatively short, ceasing within 24 to 30 hours in the absence of antibiotic therapy. Patients with cholera suffer from symptoms which are not self-resolving.

Endoscopic studies (using a triple-lumen polyvinyl tube, passed perorally under fluoroscopic control), performed on the Calcutta patients during illness, revealed large populations of *E. coli* in the proximal small intestine. Upon recovery of the patients the organisms were no longer observed (5, 48). The organisms isolated from the Calcutta patients could be placed into the serogroups O78 and O126 (5, 48). The striking similarities

TABLE 1

"O" Serogroups Associated with the Major Categories of Diarrheagenic *E. coli*

1) Enteropathogenic *E. coli* :	O55, O86, O111, O119, O125, O126, O127, O128ab, O142, O18, O44, O112, O114.
2) Enterotoxigenic *E. coli* :	O6, O8, O15, O20, O25, O27, O63, O78, O80, O85, O115, O128ac, O139, O148, O153, O159, O167.
3) Enteroinvasive *E. coli* :	O28ac, O29, O124, O136, O143, O144, O152, O164, O167.
4) Enterohemorrhagic *E. coli* :	O157, O26, O111.
5) Enteroadherent *E. coli* :	O serogroups not defined.

ref: 68

between the clinical symptoms of this group and those with cholera prompted researchers to search for an enterotoxin as the causative agent of disease. Volunteer studies with toxigenic strains of *E. coli* obtained from extraintestinal sites directly demonstrated the diarrheagenic capacity of enterotoxigenic *E. coli* in humans. Subsequently an enterotoxin with biological properties very similar to cholera toxin was identified in the cell free culture fluid of the Calcutta *E. coli* isolates (95).

It has since been established that ETEC infections are a major cause of infant diarrhea in developing countries, as well as being the agent most frequently responsible for travellers' diarrhea. (13, 40, 56, 98, 101). Such infections are acquired by the fecal-oral route. The bacteria colonize the proximal small intestine and release a heat-labile (LT) or heat-stable (ST) enterotoxin, or both (19, 20, 24, 68, 87). The production of these enterotoxins are encoded by genes contained on plasmids (99, 100, 112, 116).

The LT enterotoxin (a classical A-B toxin similar to cholera toxin) is a high molecular weight (86,500) antigenic protein, composed of an enzymatically active A subunit joined to a B oligomer consisting of five identical subunits. As with other A-B toxins, the B oligomer is responsible for binding to cell surface receptors and perhaps providing a mechanism for the A subunit to penetrate the plasma membrane. Receptors for the LT- B subunit on intestinal cell surface membranes include GM_1 ganglioside and a recently-described glycoprotein (25, 51). After binding, the A subunit enters the cell and irreversibly activates

the adenylate cyclase complex by destroying the GTPase activity of the alpha subunit of the cyclase stimulatory component(G_s). This results in the intracellular accumulation of cyclic AMP which causes overt secretions by crypt cells and decreased absorption by villus tip cells. This results in the accumulation of electrolyte-rich fluid in the lumen of the small intestine, clinically manifested as watery diarrhea (25, 51).

The other type of enterotoxin is ST, a low molecular weight (1,000 - 6,000), heat-stable, non-immunogenic polypeptide. Two forms of ST exist: STa, which is methanol soluble; and STb, which is methanol insoluble. These two forms (STa and STb) are immunologically and genetically distinct. Of the two forms, STa has been more extensively studied (25, 51).

Binding studies conducted with [^{125}I]-labeled *E. coli* STa and brush border membranes which were enzyme-treated with pronase, phospholipase A_2, phospholipase C, neuraminidase and endoglycosidase P suggested that the receptor for STa is a protein. Only pronase treatment of membranes resulted in decreased binding of [^{125}I]-labeled STa (19, 39). Once the STa is bound to the gastrointestinal epithelial cell membrane, it activates particulate guanylate cyclase activity, resulting in an intracellular accumulation of cyclic GMP (50). This cyclic nucleotide alters the cell membrane function, resulting in net secretion of water and electrolytes (86). The mechanism of intestinal secretion due to STb is unknown, for it does not appear to involve increased levels of cyclic nucleotide (20).

1.3 *E. coli* pili:

Houwink and Van Iterson (52), performed electron microscopy on bacterial flagella. In the course of their study of *E coli* , they noticed, in addition to flagella, the occasional presence of thin, rigid, fragile filaments radiating from the surface of the bacterium. It was their impression that these filaments were formed on bacteria in contact with a solid surface; hence they were an organ of attachment.

Several years later Duguid et al. (22) obtained results which supported Houwink and Van Iterson's opinion that these thin, rigid, filamentous appendages (termed "fimbriae" by these investigators) act as an organ of attachment. In the investigation of Duguid et al., it was found that strains possessing fimbriae had the ability to agglutinate red blood cells from a variety of animals.

In a subsequent investigation, Duguid and Gillies (21) noticed that the agglutination of guinea pig erythrocytes by *Shigella flexneri*, which also express pili, could be inhibited by adding D- mannose to a final concentration of 0.5%. Even if D-mannose was added after hemagglutination had occurred, the clumps would disperse on further mixing. Consequently, this type of hemagglutination was termed mannose sensitive hemagglutination (MSHA), and the fimbriae which displayed this activity were later classified as type 1 or common pili. The genes responsible for the production of type 1 pili are present on the chromosome as opposed to being on plasmids (78). Type 1 pili are expressed by many members of the Enterobacteriaceae family (14). In addition to bacteria which express type 1 pili, Duguid *et*

al (21) also identified piliated bacteria that hemagglutinated erythrocytes in the presence of D-mannose. This phenomenon was termed "mannose resistant hemagglutination" (MRHA). Duguid's investigations into bacterial hemagglutination became the basis for a simple procedure which continues to be extensively used for grouping organisms expresssing different types of pili.

MSHA by type 1 pili has been extensively studied. Firon, et al. (37) examined the interaction between mannose containing oligosaccharides and type 1 pili of *E. coli* in the yeast cell agglutination system. Yeast cells are agglutinated by type 1 piliated organisms because of their mannose containing cell walls (37). The aim of their investigation was to characterize the receptor combining site of type 1 pili. This would be crucial to understanding the mechanism by which type 1 pili adhere to cell surface receptors and may eventually aid in designing highly effective inhibitors for preventing adhesion and subsequent infection. Briefly, for each oligosaccharide examined, the concentration causing 50% binding inhibition was determined and the relative inhibiting activity (compared to that of methyl-D-mannoside) was calculated. The best inhibitors of yeast aggregation by intact bacteria, as well as by isolated pili, were the aromatic glycoside p-nitrophenyl D-mannoside and the trisaccharide Manα1->3Manβ1->4 GlcNAc. Firons' et al. results showed that the type1 pili combining site is an extended one, corresponding to the size of a trisaccharide. It contains a hydrophobic region in the form of a pocket on the surface of the lectin into which hydrophobic groups such as p-nitrophenyl might

fit. Therefore, the combining site fits best the structure found in short oligomannosidic chains present in the core region of asparagine-linked oligosaccharides on glycoproteins. In addition to type 1 pili, other examples of pili interacting with complex carbohydrate receptors on eucaryotic cells are listed in table 2.

There are two types of pilus morphology seen in *E. coli* : (1) the thin, rigid rods with diameters of approximately 7 nm, as illustrated by type 1, CFA/I, and P pili (Table2); or the thin, wavy, flexible, threads with diameters of 2 nm as exemplified by the K88 and F41 pilus structures (58).

X-ray diffraction studies of type 1 pili indicated a helical rod -like structure with 3.125 subunits per revolution and a subunit pitch distance of 2.3 nm. An axial hole with a diameter of 2 nm is located in the center. The pilus is composed of approximately 1000 identical pilin subunits, held together by noncovalent forces. The overall length of the pilus is approximately 1μm (58). Pilus X-ray diffraction studies have not been performed on the thin, flexible type pili.

In addition to pilus structure there has been considerable interest in determining whether the adhesive properties of type 1 pili (as well as other pili), is due to the pilin subunits or an adhesin molecule possibly associated with the pili and responsible for attachment. Recently, through the use of DNA technology and transposon mutagenesis, Maurer and Orndorff (78, 79) demonstrated that the hemagglutinating ability of type 1 pili requires a protein which is different from the pilin subunit. They derived mutant *E. coli* strains which expressed type 1 pili,

TABLE 2: SUMMARY OF PILI RECEPTOR INTERACTIONS

PILI TYPES	HOST STRAIN	CLINICAL CONDITION	RECEPTOR MOIETY
Type1	Broad	Urinary infections	manα1–3 manβ1–4 GlcNAc
P	Human	Human pyelonephritis	D-Gal(α1-4)D-Gal
S	Human	Sepsis & Pyelonephritis	NeuAcα2–3 Galβ1–3 GalNAc
G	Human	Acute pyelonephritis	Terminal N-acetyl-D glucosamine
Gonococcal	Human	Gonorrhea	Galβ1–3GalNAcβ1–3 Gal
K88	Porcine	Porcine diarrhea	Galactoside of unknown structure
K99	Bovine, ovine & porcine	Porcine, ovine, bovine diarrhea	Unknown carbohydrate (UC)
987P	Porcine	Porcine diarrhea	(UC)
F41	Bovine	Bovine diarrhea	(UC)
RDEC-1	Lapine	Lapine diarrhea	(UC)
CFA/I	Human	Human diarrhea	(UC)
CFA/II	Human	Human diarrhea	(UC)
PCF8775	Human	Human diarrhea	(UC)

source: 33, 37, 55, 58, 63, 71, and 72

morphologically and serologically identical to wild type, but were unable to hemagglutinate guinea pig erythrocytes. The hemagglutination property was mapped to a chromosomal locus, by genetic techniques and was termed pil E. The pil E gene is not part of the region that encodes type 1 pilin. The result of this investigation indicated that the pil E locus specifies the expression of at least one *trans* -acting product which is necessary for piliated cells to cause hemagglutination.

Similar results have been demonstrated for P pili by Lindberg, et al. (72). They reported that the adhesin protein, mediating the binding to digalactose-containing glycolipids (see Table 2) present on urinary tract epithelial cells, is encoded separately from the gene encoding the P pilin molecule.

ETEC pili display certain common features: They consist of either rigid structures 6-7 nm in diameter or of wiry, flexible structures 2-3 nm in diameter; are encoded by plasmids that usually also encode the heat-stable enterotoxin and often the heat-labile enterotoxin (58, 68); consist of protein subunits, ranging in size from 14 to 22 kilodaltons (Kdal) and are expressed at 37°C but not at 18°C. The molecular mechanism for the temperature-dependent expression may be at the transcriptional level and rely on a temperature-sensitive repressor molecule. Alternatively, the membrane lipids of *E. coli* may partially solidify at the lower temperature, thereby inactivating the pilus translocation system(s) (58). Other common characteristics of ETEC pili include serotype restriction of pilus expression, that is to say that a particular type of pilus is

expressed only in a few types of *E. coli*, a phenomenon which is not yet understood. In addition, agglutinated bacteria can be eluted from erythrocytes at 37° C, and the hemagglutinating activity is destroyed by heating the bacteria at 65° C for 30 minutes (68). Most importantly, ETEC pili are responsible for mediating MRHA.

Normally, *E. coli* do not colonize the small intestine to an appreciable extent (5, 48, 64, 65). However, ETEC strains which express pili are able to resist the mechanism of peristalsis and subsequently adhere to these surfaces (43). A variety of pilus types have been identified in ETEC isolated from various animal sources (see Table 2). However, in addition to pili, afimbrial adhesins have been identified in human ETEC (strains 444-3, 469-3 and 2230; 16, 38, 114).

1.4 Colonizing Factor Antigen:

The first pilus colonization factor associated with a human ETEC strain was described by Evans, et al. (30). They identified an ETEC strain, designated H10407, serotype O78:K80:H11, which was isolated from a patient in Bangladesh. The organism contained a plasmid-associated heat-labile surface antigen that facilitated colonization of the small intestine. Hence, it was termed "colonization factor antigen" (CFA). It was subsequently named CFA/I when a second colonization factor antigen (CFA/II) was identified and found to be immunologically distinct (27).

Immunodiffusion studies have shown that CFA/II is comprised of more than one antigenic component. These

components are called *E. coli* surface asociated antigens CS1, CS2, and CS3. In a large number of ETEC strains examined, the antigens seemed to occur in pairs (ie., CS1 and CS3 or CS2 and CS3) but CS2 only positive strains have also been identified (15).

CFA/I pili are encoded on a single non-autotransferring plasmid with a molecular weight of approximately 60 x 10^6. The same plasmid also encodes the heat-stable enterotoxin (ST). Further, there is approximately 90% homology in DNA between the 60 x 10^6 plasmid obtained from different O78 ETEC strains isolated from different geographic locations (30, 81, 99, 115, 116). The sequences required for the production of CFA/I pili are located on two separate regions of the plasmid, designated regions 1 and 2 (6 and 2.1 kbp, respectively;116). Region I encodes the structural gene for the CFA/I pilus subunit. CFA/I pilin is synthesized as a larger precursor molecule which undergoes processing, resulting in the smaller mature form. The products encoded by region 2, although not essential for subunit production, are required for pilus assembly (116). The organization of CFA/I pilin genes is in contrast to the K88 and K99 pili systems (from ETEC of animal origin). Both K88 and K99 pili systems contain the genes necessary for the expression of their pili on a single transcriptional unit of approximately 7 kbp. Five gene products are required for K88 pili expression and seven gene products for K99 expression (43, 58).

The pilins from K88 and K99 are well characterized. The K88 pilin exists in several antigenic variants, which are K88ab, K88ac, and K88ad. The common antigenic factor, consisting of one

or more antigenic determinants is termed "a", whereas the variable factors have been denoted "b", "c", and "d". The K88 pilin subunit has been sequenced and consist of 264 amino acids, with a molecular weight of approximately 27,500. It has an isoelectric point of 4.2 (43, 58). In contrast, the K99 pilus subunit consists of 159 amino acid residues, with a molecular weight of 18,400. In addition, K99 pili have an isoelectric point of 9.5, which is believed to promote binding to the predominantly negatively-charged epithelial cell surface. The K99 pili subunit is also held together by disulfide bridge links (43, 58).

The CFA/I pilin protein has been well characterized. Intact pili have an average molecular weight of 1.6×10^6, as determined by equilibrium centrifugation (28). The isoelectric point of CFA/I pili is pH 4.8 (40). The primary structure of the pilin subunit consists of 147 amino acid residues with a molecular weight of 15,058. The amino acid sequence does not reveal any homology with the known sequences of other pili (57, 59). However, the protein contains a rather hydrophobic carboxyl terminus (similar to the K88 pilus protein), as determined by the hydrophobicity index and the absence of charged amino acids among the last 23 residues. It has been proposed that this region of the molecule links subunits by hydrophobic interactions, thereby helping to maintain the integrity of the fimbrial superstructure (57, 59). The protein contains no cysteine groups, indicating that no disulfide bridges link the residues together. The resultant polymeric, filamentous organization of pilin subunits may provide an array of binding sites for specific receptors on host epithelial cells and

erythrocytes, as well as localization of potential antigen determinants.

Potential antigenic regions of CFA/I were predicted by Klemm and Mikkelsen (59). The methodology of Hopp and Woods (utilizing algorithms to assign hydrophilicity values to each amino acid in a sequence) and the empirical algorithm of Chou and Fasman (which is based on a statistical treatment of secondary structure results obtained by X-ray crystallographic investigations of 29 proteins), enabled the prediction of six regions of potential antigenicity. These sites, listed in descending order of antigenicity, are located in residues 52-57, 61-66, 37-42, 112-116, 19-24 and 92-99.

Electron micrographs of CFA/I pili revealed filamentous, structures with diameters of approximately 3 to 6 nm. The length is variable, possibly because pili are subjected to shearing during preparation for electron microscopic examination. CFA/I pili exhibit a tendency to aggregate and form bundles, depending upon the pili concentration (28).

Although the role of CFA/I pili in mediating attachment to human intestinal cells and erythrocytes is well documented, the biochemical identity of CFA/I receptor(s) has not yet been conclusively established (29, 31, 32, 33, 35, 47, 49, 61, 62). Initially, Evans, et al. (31) suggested that the surface moieties relating to the ABO erythrocyte antigen typing system may be involved in the hemagglutination reaction. They implied that the immunodominant sugar, N-acetyl-D-galactosamine, involved in blood type A specificity, is associated with the hemagglutination

reaction (31). Subsequently, Faris, et al. (34, 35), suggested that the oligosaccharide units of ganglioside GM_2 could be the erythrocyte receptor for the CFA/I pili. Recently, Bartus, et al. (6), stated that the human erythrocyte receptor for CFA/I pili is probably a sialoglycoprotein.

1.5 Specific Aims of the Research:

The objective of the present investigation was to identify erythrocyte receptors for CFA/I pili. I chose human erythrocytes rather than intestinal epithelial cells because: (1) the large quantities of cells required for receptor purification are more easily obtained from blood than from human intestinal biopsy material; (2) receptors have not been identified in tissue culture cell lines. Moreover, since continuous cell lines may possess altered membrane components, structures serving as receptors may not be identical to those found in native intestinal cells; (3) my investigations would be greatly facilitated by the amount of structural information available on the major components of the human erythrocyte membrane and could build upon the results of investigations of other important pili erythrocyte interactions; and (4) Receptors for type 1, P, S and G pili have been identified in erythrocytes (35, 55, 63, 73, 85, 88).

However, the relationship between the red blood cell receptor and intestinal receptor for CFA/1 pili is uncertain because hemagglutination does not occur at 37° C (26, 43). This observation suggests that : (1) the CFA/1 pilus may contain two independent receptor binding domains on one or more adhesins,

each strictly recognizing different receptors - one site for intestinal cells receptors, the other for receptors on erythrocyte membranes; or (2) one binding site which is flexible enough to recognize more than one receptor but the "fit" for the intestinal cell receptor is better than that of the erythrocyte receptor. It will only be possible to distinguish between these two possibililties when erythrocyte and intestinal cell receptors have been completely identified and characterized.

2. MATERIALS AND METHODS

2.1 Materials

CFA agar materials: casamino acids, yeast extract and agar were purchased from Difco Laboratories, Detroit, Michigan, USA; whereas, the magnesium sulfate and manganese chloride were purchased from Fisher Scientific, Edmonton, Alberta, Canada

L-broth materials: tryptone and yeast extract were purchased form Difco Laboratories, Detroit, Michigan, USA; whereas, the sodium chloride was purchased from BDH Chemicals, Toronto, Ontario, Canada.

The tryptose used for making nutrient agar plates (TSA) was purchased from Difco Laboratories.

The chemicals used for making PBS: potassium chloride, potassium phosphate monobasic, sodium phophate dibasic heptahydrate, calcium chloride and magnesium sulfate were purchased from Fisher Scientific, Edmonton, Alberta, Canada; whereas, the sodium chloride was purchased from BDH Chemicals, Toronto, Ontario, Canada. The mannose that was added was bought from Sigma Chemical Company, St. Louis, USA.

The reagents use in the Birnboim-Doly plasmid prep: trizma base, and ethylenediamine tetraacetic were purchased from Sigma Chemical Company. Glucose, sodium glutamate, and sodium acetate were bought from Fisher Scientific. Lysozyme (from hen egg white) and ribonuclease (RNase, from bovine pancreas) were purchased from Boehringer Mannheim, Dorval, Quebec, Canada.

The sodium dodecyl sulfate was bought from BioRad, Richmond, California, USA.

The TAGS (Trizma, Azide, Glutamate, and Saline) buffer reagents: trizma base, and trizma hydrochloride were purchased from Sigma Chemical Company, St. Louis, USA; the sodium glutamate and sodium azide were purchased from Fisher Scientific; and sodium chloride was bought from BDH Chemicals.

The proteinase K (digest for LPS, from *Tritirachium album*) was purchased from Boehringer Mannheim.

The electron microscopy materials: phosphotungstic acid (PTA), grids, osmium tetroxide, ruthenium red, uranyl acetate, propylene oxide, beem capsule, glass stripes, epon, 2-dimethylaminoethanol (DMAE) (epoxy accelerator), nadic-methyl anhydride (NMA) (epoxy hardener), and lead citrate were purchased from Fisher Scientific; the microtone ultracut was purchased from Reichert-Jung Scientific Instruments, Belleville, Ontario, Canada; formvar was bought from Ernst F. Fullanm Inc. Schenectady, N. Y. USA; the glutaraldehyde was order from Ladd Research Industries through Ingram and Bell Scientific, Edmonton, Alberta, Canada; and the LKB knife maker was purchased from Fisher Scientific.

The materials for the isolation, purification quantification and analysis of CFA/I pili "preps": 150mm x 15mm plates, ammonium sulfated, folin-ciocalteau, the verre borosilicated test tubes (12mm x 75mm) and glycerin were purchased from Fisher Scientific; the cesium chloride was purchased from Terochem Laboratories Ltd., Edmonton, Alberta, Canada; the acrylamide,

bisacrylamide, ammonium persulfate, coomassie brilliant blue R-250, sodium dodecyl sulfate (SDS), SDS-PAGE MW markers (range 10,000-100,000), tetramethylethylenediamine (TEMED), and the SDS-PAGE apparatus were purchased from BioRad Richmond, California, USA; the glycine, ethylenediamine tetraacetic acid (EDTA), trizma base, and trizma hydrochloride were bought from Sigma; and the 2-mercaptoethanol was purchased from Eastman Kodak Company, Rochester N. Y. USA. The bromophenol blue was purchased from Fisher Scientific. The peristaltic pump was purchased from Pharmacia, Dorval, Quebec, Canada. And the Ultra clear centrifuge tubes were purchased from Beckman Instruments Inc., Palo Alto, California, USA.

The following reagents were used to silver stain the LPS in the polyacrylamide gel: periodic acid, sodium hydroxide, silver nitrate, and paraformaldehyde. These chemicals were purchased from Fisher Scientific. The ammonium hydroxide was bought from J. T. Baker Chemical Co., Phillipsburg, N. J., USA.

The enzymes and reagents used in the treatment of human erythrocytes were bovine pancreatic trypsin type III, 12,400 BAEE (Nα-benzoyl-L-arginine ethyl ester) units/mg protein, neuraminidase (typeX, 160 N-acetylneuramin-lactose units/mg protein) from *Clostridium perfringens* , and phenyl methylsulfonylfluoride were obtained from Sigma Chemical Company. Dimethyl sulphoxide was purchased from BDH Chemicals.

The reagents used to prepare erythrocytes membranes and the extraction of glycoproteins: sodium chloride, purchased from

BDH Chemicals; trizma base, ethylenediamine tetraacetic, and phenylmethylsufonyl fluoride was purchased from Sigma Chemical Company; dimethyl sulfoxide (DMSO), was bought from BDH Chemicals; the sodium phosphate, phenol, the 15mL conical test tubes, and the dialysis tubing (MW cut off 6,000-8,000) were purchased from Fisher Scientific; the lithium 3,5 diiodosalicylate was purchased from Eastman Kodak Company, Rochester, N.Y., USA.

The materials for the iodination protocol: radioactive sodium iodide was purchased from Edmonton Radiopharmaceutical Centre, Edmonton, Alberta, Canada; Iodo-gen was obtained from the Pierce Chemical Co., Rockford, Il., USA; the pasteur pipets, trichloroacetic acid and polystyrene test tubes were purchased from Fisher Scientific; cysteine and bovine albumin were purchased from Sigma Chemical Company; and sephadex G-25 was purchased from Pharmacia.

The few reagents use in the polystyrene test tube binding assay: glutraraldehyde was purchased from Ladd Research Industries through Ingram and Bell Scientific; lysine was bought from Sigma Chemical Company; sodium carbonate and the polystyrene test tubes were purchased from Fisher Scientific.

The *Mycopasma gallisepticum* lectin was purchased from Sigma Chemical Company. The round bottom well microtitre plate were bought from Flow Laboratories Inc., Mississauga, Ontario, Canada.

The protein A gold was purchased from SPI supplies, Toronto, Ontario, Canada.

The reagents use in the preparation of colloidal gold: polyethylene glycol (PEG), and chloroauric acid were purchased from Sigma Chemical Company; the sodium citrate was bought from Fisher Scientific.

The wheat germ agglutinin-agarose was purchase from Pharmacia, and the N-acetylglucosamine was bought from P. C. Biochemicals Inc., Milwaukee, Wis., USA.

2.2 Characterization of bacterial strains

The enterotoxigenic *Escherichia coli* (ETEC) strains H10407 (078: H11; CFA/I$^+$) and H10407P (078: H11; CFA/I$^-$) a spontaneous mutant lacking CFA/I pili, were kindly provided by Dr. M. C. Finlayson (Provincial Laboratory of Public Health, Edmonton, Alberta). Both strains were grown overnight at 37°C, (and maintained at 4° C) on CFA agar (see Appendix 1 for details) as described by Evans et al. (31), to enhance expression of CFA/I pili and suppress the production of type 1 pili (30, 62).

The presence of the 60 x 10^6 MW plasmid (30), which contains the genes encoding CFA/I pili (in the H10407 CFA/I$^+$ strain) was confirmed by agarose gel electrophoresis following the alkaline extraction procedure devised by Birnboim and Doly (8). This method takes advantage of the resistance of plasmid DNA molecules to strand separation by alkali. Briefly the procedure consists of removing the outer membrane and cell wall of the gram negative organisms with EDTA and lysozyme (a loopful of bacteria in late exponential growth, was suspended in 100 µL of solution 1 (see appendix 4) for 10 minutes on ice), followed by the

lysis of the resulting spheroplasts (200µL of solution 2 (see appendix 4) was added and incubated on ice for another 5 minutes) and the solubilization of the proteins under alkaline conditions with a solution of 4% SDS in 0.05M Tris-hydrochloride and 0.01M EDTA at pH 12 (pH values higher than 12.5 will irreversibly denature the plasmid DNA, therefore it is important to measure the pH accurately).

The separation of the nucleic acid material from cellular debris was accomplished by precipitating denatured proteins, cell membrane and some chromosomal DNA in 3M sodium acetate, pH 4.8 (150µL of sodium acetate was added and left on ice for 10 minutes). The chromosomal/plasmid DNA was further purified by phenol/chloroform extraction, then precipitated by the addition of 95% ethanol (1 mL). The precipitate was then suspended in 30-50 µL of Tris-EDTA buffer, pH 8.0, and analyzed on a 0.7% Tris-borate, pH 8.8 agarose gel after electropheresis for 8.5 h at 100V (constant).

In addition, the strains were routinely examined for the presence or absence of pili by a Philips 300 electron microscope (staining with 1% sodium phosphotungstate), which revealed that greater than 70% of the H10407 (CFA/I+) bacteria per grid were multipiliated (more than 50 pili/cell), whereas no pili could be detected on the H10407P strain. Moreover, because both strains are known to produce type 1 pili, I also checked for mannose resistant and mannose sensitive hemagglutination of guinea pig and human erythrocytes. The identity of the pili was further confirmed by bacterial agglutination tests using polyclonal and

monoclonal anti-CFA/I antibodies (both the polyclonal and monoclonal antibodies were a gift from Dr. W. Paranchych, Dept. of Biochemistry, University of Alberta).

2.3 Purification of CFA/I pili

Confluent growth of H10407 was obtained overnight at 37° C on CFA agar, in 150mm x 15mm plates (117), (Fischer Scientific, Edmonton, Alberta. The bacteria were scraped from the agar surface and suspended in cold phoshate buffered saline (PBS) by mixing with a magnetic stirrer at 4° C for 30 minutes. To ensure that the bacteria were piliated, a slide hemagglutination test was performed by mixing an aliquot of bacterial suspension with an aliquot of human erythrocytes, diluted 1:10 in PBS (at a concentration of 10^8 cells/ml) containing 0.4M mannose. If hemagglutination was observed the pili were sheared off the bacteria by treating the suspension for 5 minutes in a stainless steel blender immersed in ice. The resulting suspension was then centrifuged at 10,400 x g for 20 minutes and the supernatant solutions were collected and pooled. Ammonium sulfate crystals (25%, w/v) were added to the resulting supernatant solution and allowed to dissolve by mixing for 30 minutes at 4° C. The solution was left overnight at 4° C in order to precipitate the pili and flagella. The following day, the precipitate was sedimented by centrifugation at 10,400 x g for 20 minutes. The supernatant solution was discarded, and the pellet was suspended in 50 mL of double distilled water (dd H_2O) and dialysed overnight against 1L of dd H_2O, using cellulose dialysis tubing with a molecular weight

cut off of 6,000-8,000. After dialysis, which included at least 2 changes of double distilled water, the pili suspension was layered onto a cesium chloride step gradient (see appendix 7 for details) and centrifuged in a Beckman ultracentrifuge for 16 hours at 98,000 x g in a SW 27 rotor.

At the end of the centrifugation procedure, two bands were observed in the cesium chloride gradients. Electron microscopic examination revealed that the top band contained the CFA/I pili and the bottom band contained flagella. This was subsequently confirmed by SDS-PAGE analysis. The top band was removed from the gradients and dialyzed against 2 liters of dd H_2O (several changes) overnight using cellulose dialysis tubing with a molecular weight cut off of 6,000-8,000. The protein concentration of purified pili preparations was determined by the Lowry method and an aliquot was analysed by discontinuous SDS-PAGE on a 14% separating gel by the Laemmli procedure (66) to determine its purity and to confirm the molecular weight of the CFA/I pili subunit.

2.4 Analysis of Lipopolysaccharides

Bacteria were grown overnight at 37°C on CFA agar plates. The following day, the strains were suspended in 4 mL TAGS buffer to an optical density of 0.12 - 0.15 (at a wavelength of 540 nm using a Coleman spectrophometer). Aliquots of 1.5 mL of the suspension were centrifuged in an Eppendorf centrifuge for 5 minutes and the supernatant solutions were removed. Fifty microliters of Laemmli's sample buffer was added to the

sedimented bacterial pellets. The samples were then sonicated 10 seconds and shaken for a further 10 seconds on a vortex mixer for to suspend the cell pellets. The suspensions were heated for 5 minutes in boiling water. They were shaken and sonicated and 10 µL aliquots of *Proteinase K* (1 mg/mL) were added. The samples were then incubated at 56° C for 3 hours, with vigorous shaking every 30 minutes. Finally, an aliquot of each sample was placed onto a 14% SDS- polyacrylamide gel, subjected to electrophoresis and silver stained .

2.5 Preparation of human erythrocytes

Human blood (packed erythrocytes) was obtained from the Edmonton branch of the Canadian Red Cross Society. Prior to use the erythrocytes were suspended in PBS (containing 1 mM Ca^{++} and 10 mM Mg^{++}) and sedimented by low speed centrifugation (500 x g for 5 minutes at room temperature). The supernatant solution and buffy coat were carefully removed from the erythrocytes and discarded and the erythrocyte pellets were suspended in PBS. The washing procedure was repeated at least twice, or until the resulting supernatant solution was clear. The washed erythrocytes were suspended in PBS at a concentration of 10^8 cells/ml as determined by hemocytometer count.

2.6 Enzymatic treatment of erythrocytes:

The erythrocytes were treated with trypsin (1 mg/ml in PBS) or neuraminidase (1 µg/ml in PBS) for 60 minutes at 37° C. Trypsin was then inactivated by adding 100 x molar excess of

phenylmethylsulfonyl fluoride (dissolved in dimethyl sulfoxide). Following enzyme treatment, the erythrocytes were washed extensively by the centrifugation procedure described above to remove residual trypsin or neuraminidase activity.

2.7 Extraction of human erythrocyte membranes with lithium diiodosalicylate:

Erythrocyte membranes were prepared by hypotonic lysis according to the procedure of Steck et al., (104). The procedure consisted of washing erythrocytes with an equal volume of PBS in 50 mL tissue culture tubes (all steps were performed at 4° C). This was accomplished by sedimenting the cells by centrifugation (500 x g for 10 min.), and discarding the supernatant solution and buffy coat. This step was repeated until the resulting supernatant solution was clear. The washed erythrocytes were then suspended in 8 volumes of freshly prepared SPIE buffer (5mM sodium phospate (pH 7.9), 1mM EDTA, and 1mM phenylmethylsulfonyl fluoride), and allowed to stand on ice for 20 minutes. The resulting membranes were sedimented (12,100 X g for 20 min.), and washed with SPIE buffer until the resulting pellet was a light tan color. At this point, the pellet was washed twice in 50mM Trizma hydrochloride, pH 7.0-7.5, and after the final centrifugation step (12,100 x g for 20 min.), the pellet was suspended in PBS buffer and stored at -70° C. The protein concentration of the membrane preparations was determined by the Lowry procedure.

The isolation of glycoproteins from the membranes was performed according to the method of Marchesi and Andrews, (76). Erythrocyte membranes (15 mg - 25 mg/mL of protein as determined by Lowry method) were added to 0.3 M lithium diiodosalicylate in 0.05 M Tris-HCl, pH 7.5. The resulting solution was mixed for 15 minutes at room temperature, at which time it was diluted with two volumes of dd H_2O and mixed for an additional 10 minutes at 4°C. The solution was subsequently centrifuged (45,000 x g for 90 minutes at 4°C), to sediment the insoluble fraction. The supernatant solution, containing the membrane proteins, was removed and mixed with an equal volume of freshly prepared 50% phenol in dd H_2O. This solution was stirred for 15 minutes at 4°C, then centrifuged at 4,000 x g for 1 hour at 4°C. The aqueous (upper) phase, containing the soluble glycoproteins, was removed and dialyzed (using cellulose dialysis tubing with a molecular cut off of 6,000-8,000), for 36 hours at 4°C against 2 L of dd H_2O (with several changes of water). The dialysed solution was then freeze-dried and suspended in cold (-20° C), 100% ethanol and mixed for 1-2 hours at 4° C . The precipitate was sedimented by centrifugation at 12,100 x g, for 30 minutes at 4° C. The ethanol washing and centrifugation steps were repeated 3 times. After the final wash, the precipitate was suspended in dd H_2O and dialyzed using cellulose tubing with molecular cut off 6,000-8,000, against 2L of dd H_2O overnight at 4° C. The clear supernatant solution containing soluble glycoproteins was retained. The glycoprotein extract fraction was stored in aliquots at -70° C for several months with no apparent

loss of activity. During the course of the investigation, the extract could be stored at 4° C for several months with no apparent loss of activity.

2.8 Live bacteria binding assay:

After overnight growth at 37° C on CFA/I agar (9 cm diameter petri plates), the bacteria were gently suspended in 1 mL of PBS so as not to remove too many pili from the cells. The bacterial cell concentration was determined in a Petroff-Hausser bacterial counting chamber and the cells were then diluted to 3-5 x 10^6 cells/mL in PBS. Two hundred microliters of the bacterial suspension were mixed with 200 µl of red blood cells (4 x 10^7 cells/mL) and incubated for 60 minutes at room temperature. The mixture was then centrifuged at 500 x g for 5 minutes to sediment the erythrocytes and bound bacteria. The supernatant solutions containing unbound bacteria were discarded. Bound organisms were released from the erythrocyte surface by incubating the cells in 200 µL of 0.05% trypsin for 30 minutes at 37° C. The mixture was then diluted in chilled L-broth to reduce the concentration of bacteria and aliquots were spread on nutrient agar plates to determine the number of bound organisms. The number of organisms in the inoculum was determined in a similar fashion and the number of bound bacteria was expressed as a percentage of those added to the binding reactions.

The assay was also performed in the presence of purified CFA/I pili (0.7 mg/ mL) to demonstrate the ability of purified CFA/I pili to compete for binding of H10407 CFA/I+ organisms.

2.9 Iodination Procedure

Purified CFA/I pili or erythrocyte glycoprotein were labeled by the iodination procedure described by Spivak et al. (102). This procedure labels aromatic amino acids like tyrosine. Briefly, 10 MBq (approximately 0.3 mCi) Na [^{125}I] was added to 5 µg of protein in 100 µL of dd H_2O. The solution was placed in an Iodo-Gen-coated, 12 x 75 mm glass test tube (Iodo-Gen was obtained from the Pierce Chemical Co., Rockford, Il). CFA/I pili soutions were exposed to Iodo-Gen for 1 minutes whereas the erythrocyte extracts required a 5 minutes reaction time for efficient iodination. After the iodination reaction was complete the solutions were filtered through a glass wool plugged Pasteur pipette into a clean 12 x 75mm glass test tube. One hundred uL of cysteine solution (1mg/mL in dd H_2O) was passed through the filter into the mixture to reduce elemental iodine (I_2) and inhibit any further iodination. The percentage of [^{125}I] counts incorporated into the pili or extract was determined by TCA precipitation (as described below) and the specific activity (MBq/µg) was calculated.

Two hundred µL of dd H_2O (containing 0.1% BSA in dd H_2O) were added to the remainder of the mixtures to prevent nonspecific binding of labeled proteins to Sephadex. The mixture was then passed through a disposable Sephadex G-25 column (1 cm x 10 cm) equilibrated with 0.1% BSA in dd H_2O to separate the [^{125}I]-labeled proteins from free [^{125}I]. Sixteen 0.5 mL fractions were collected and 5 µL aliquots of each fraction were counted in

an LKB Rackgamma II 1270 gamma counter (counting efficiency 50% for [^{125}I]), to determine the location of the fractions containing labeled protein. The concentration of iodinated protein in the G-25 peak fractions was calculated from the total TCA precipitated counts recovered in the void volume fractions and the specific activity. In a typical experiment, 1.5 mL contained approximately 4 ug iodinated protein. Specific activity was approximately 8 x 10^7 cpm/μg.

2.10 Determination of [^{125}I] Incorporated into Protein by TCA precipitation

Ten μL of iodinated protein was mixed with 1 mL 1% BSA in dd H_2O in a 12 x 75 mm glass test tube. This was performed in duplicate. One-half mL of 10% TCA was added to one sample and 0.5 mL of dd H_2O was added to the other sample. Both tubes were agitated and incubated on ice for at least 10 minutes to allow the precipitation reaction to go to completion. Next, the tubes were centrifuged at 700 x g for 10 minutes to sediment the precipitated protein in the TCA-containing tube. The amount of ^{125}I radioactivity in suitable aliquots of the resulting supernatant solutions was determined in the gamma counter. The percentage of radioactivity precipitated by TCA was determined from the difference between the number of counts remaining in the TCA supernatant solutions and those in equivalent aliquots in the unprecipitated control tubes. All binding experiments were performed exclusively with preparations containing greater than or equal to 70% TCA precipitable counts.

2.11 Polystyrene Test Tube Binding Assay

Prior to attaching purified CFA/I pili or extract, the 12 x 75 mm polystyrene tubes were activated with 1 mL of 2.5% glutaraldehyde in deionized water for 24 hours at 4°C. The glutaraldehyde-treated tubes were subsequently washed with deionized water to removed excess glutaraldehyde. Next, 1 mL of protein (ranging in concentration from 2 - 100 µg), in coating buffer (0.1M sodium carbonate, pH 9.6) was added and the tubes were incubated at 4°C for a further 18 hours. The protein coated test tubes were then washed 3 times with 1 mL aliquots of water containing 0.1% BSA. To ensure that all of the remaining glutaraldehyde groups had been neutralized, 1mL of 0.5M lysine was added and the tubes were incubated for an additional hour. The test tubes were then washed as described above. Binding assays were performed in 1 mL dd H_2O containing 0.1% BSA and iodinated proteins (10 - 100µg). The assay tubes were incubated at room temperature for 3 hours; the binding mixtures were then removed by aspiration and the tubes were washed with 1 mL of 0.1% BSA (in dd H_2O). The amount of bound radioactivity was determined in an LKB Rackgamma 1270 gamma counter and converted to ug bound protein using the specific activity values calculated for each lot of iodinated protein.

2.12 Binding Assay Using Labeled Extract and Live Bacteria

The agar grown bacterial strains were gently suspended in 1 mL sterile dd H_2O as described earlier. An aliquot of the

suspension was then diluted to 5 mL with sterile dd H_2O and adjusted to an optical density of 0.12-0.15 at 540 nm using a Coleman spectrophometer. Next, 1 mL of this suspension was mixed with 50 µL of [^{125}I]-labeled extract (approximately 5x10^6 cpm) diluted 10X in 0.1% BSA dd H_2O and incubated at room temperature for 1 hour, in 1.5 ml microcentrifuge test tubes. The bacteria were sedimented by centrifugation (3 minutes) in a Eppendorf microcentrifuge. The supernatant solution was removed, the bacterial cell pellet was suspended in dd H_2O and the centrifugation step was repeated. Finally, 100 µL of Laemmli's SDS-PAGE sample buffer (see appendix 2) was added to the washed pellet of bacteria, and the suspensions were heated for 5 minutes in boiling water and loaded onto a 14% SDS-polyacrylamide gel. The gel was stained with Coomassie Blue, dried on a Bio-Rad model 1125B slab gel drier and exposed for 48-72 hours at -70° C to Kodak X-OMAT RP film using DuPont Cronex Lightning Plus intensifying screens. Densitometric analysis of the autoradiograms was performed on a Chromoscan 3 densitometer (Dept. of Biochemistry, University of Alberta) from the Joyce Loebl, Co.

Competitive binding assays were also performed. One mL of purified CFA/I pili (1.54 µg/µL) was mixed with 50 µL of labeled extract (diluted 10X in 0.1% BSA ddH_2O) and incubated at room temperature for one hour, in 1.5 mL microcentrifuge test tubes. Then pelleted bacteria resulting from the sedimentation of 1 mL of bacterial suspension (optical density of 0.12-0.15 at 540 nm) was added to the 1 mL of purified CFA/I pili containing the

labeled extract. This mixture was incubated for another hour. The bacteria were then sedimented by centrifugation (3 minutes) in an Eppendorf microcentrifuge. The supernatant solution was removed and the bacterial cell pellet was washed twice with ddH_2O as described above. The bacterial cell pellets were then analysed by the SDS-PAGE procedure.

2.13 *Mycoplasma gallisepticum* Lectin Hemagglutination Assay

Fifty microliters of purified CFA/I pili (0.5 mg/mL, in PBS) were added to washed human erythrocytes (4 x 10^5 cells/mL in 50 µL of PBS containing 0.4 M mannose) in a 96 round-bottom well microtitre plate. After 1 hour incubation at room temperature, with occasional rocking to keep the erythrocytes suspended and facilitate pili binding to receptors, 50 uL of *M. gallisepticum* lectin, (two-fold serial dilutions with PBS containing 0.4M mannose), was added to the appropriate wells. The microtitre plate was examined for hemagglutination inhibition after 2 hours at 4°C.

The controls for this assay were the following: (1) wells containing only erythrocytes; (2) wells containing erythrocytes and purified CFA/I (50 uL at 0.5 mg/mL); and (3) wells containing erythrocytes and two-fold dilutions of *M. gallisepticum* lectin.

2.14 Protein A Gold Labeling

This procedure was conveniently carried out on a sheet of Parafilm (10 x 25 cm) stuck to the bench top using a film of water. First, the grids (formvar- and carbon-coated; glow discharged

before use with a Denton, Vacuum DV-502A apparatus) were floated upside down for 20 minutes on a PBS droplet (with 0.5% BSA) containing the antigen sample. The grids were then picked up with a platinum loop and excess solution was carefully removed with a small strip of blotting paper. Grids were washed three times by floating them on droplet of 0.5% BSA in PBS for a few seconds with blotting between washes. The grids were transferred to an antibody droplet, diluted in 0.5% BSA in PBS (for antisera with high titres, an optimal dilution was found to be approximately 1:100) and incubated at room temperature for about 1 hour. An inverted Petri dish was used to cover the droplet(s) to reduce evaporation during the incubation period. After incubation, the grids were blotted dry and washed in 0.5% BSA in PBS as described above and floated on a droplet of protein A gold (diluted 1/100 in 0.5% BSA-PBS) under cover for 1 hour at room temperature. Subsequently, the grids were blotted, washed, and stained with 1% sodium phosphotungstate. They were blotted dry with small pieces of filter paper before examining them in the Philips EM 300 transmission electron microscope using an accelerating voltage of 60 KV.

2.15 Electron Microscopy

The two bacterial strains (CFA/I$^+$ and CFA/I$^-$) were mixed with human erythrocytes (in a 1:10 ratio respectively) and incubated at room temperature for 1 hour. The cell suspension was then centrifuged to sediment the erythrocytes and bound bacteria (for further details see section 2.8). The supernatant

solution was removed and the sedimented cells were fixed overnight at 4°C with 3.5% glutaraldehyde in 0.1M phosphate buffer, pH 7.2 in the presence and absence of 0.075% Ruthenium Red. The next day the fixed cell pellets were washed 3 times (15 minutes each) in phosphate buffer and post-fixed with OsO_4 (in phosphate buffer, pH 7.2) for 1-2 hours. The specimens were rinsed in phosphate buffer 3 times, each for 15 minutes, and then dehydrated in ethanol in a stepwise gradient from 25% to100%, 15 minutes at each concentration. Absolute ethanol was then replaced by propylene oxide for 15 minutes. The propylene oxide was replaced with Epon diluted 1:1 with propylene oxide and left overnight in open vials for the propylene oxide to evaporate. The next day, the specimens were transferred to fresh Epon and left for 2 hours. Finally, the specimens were transferred to fresh Epon in BEEM capsules and were left overnight at 60° C to allow polymerization of the Epon. The specimens were trimmed and sectioned on a ultramicrotome, the sections were stained with uranyl acetate and lead citrate (10 & 3 minutes, respectively), examined and photographed with the Philips EM 300 transmission electron microscope.

2.16 Preparation of Colloidal Gold

All glassware used in this preparation was chromic acid-washed, well rinsed in hot tap water and autoclaved prior to use to remove contaminants. Small quantities of contaminants cause cloudiness of the gold preparations or interfere with the formation of the desired size of gold sphere (44). All aqueous solutions

required in this preparation were made with dd H_2O processed through a MilliQ water treatment system (mQ H_2O).

Colloidal gold was made by reducing chloroauric acid ($HAuCl_4$) with sodium citrate (41). Five mL of 0.1% $HAuCl_4$ in mQ water was added to 45 mL of mQ water and the solution was stirred continuously and heated to the boiling point. 0.75 mL of freshly-prepared 1% sodium citrate was then added, yielding a gold particle size of approximately 24 nm. The solution was heated to 100° C with constant stirring. When the solution turned a burgundy color it was allowed to cool to room temperature and 1 mL 1% aqueous polyethylene glycol (PEG 20,000) was added to stabilize the colloidal gold suspension (44, 91). The pH of an aliquot of the solution was measured and the stock solution was adjusted to pH 4.7 with 1M acetic acid.

2.17 Preparation of "Glycoprotein Extract": Gold Complexes

Adsorption binding equilibrium experiments were used to determine the minimum amount of protein required for stabilization of the colloidal gold (44, 91, 92). From a stock of 1 mg/mL of glycoprotein extract, two-fold dilutions, ranging from 0.00195 to 0.5 mg/mL, were prepared. Two mL of each dilution was added to 0.2 mL of colloidal gold solution and vigorously mixed. After 5 minutes, 0.2 mL of 10% NaCl was added, mixed rapidly and allowed to stand 5 minutes before determining the optical density at 580 nm. Protein-colloidal gold solutions differ in color as their concentration ratio changes, from pink to clear to purple-blue. A pink color indicates protein excess. A clear

solution occurs when the ratio of protein extract to colloidal gold is approximately equivalent. A purple-blue solution indicates an excess of colloidal gold particles. To ensure complete coating of the colloidal gold particles, a protein concentration two times in excess of the equivalent point is sufficient.

It was calculated that 1.25 mg of extract could stabilize 18.75 mL of colloidal gold (pH 4.7). The glycoprotein extract was added dropwise to the colloidal gold with gentle stirring. After 5 minutes, 2 mL of 1% PEG was added to prevent aggregation of the extract-colloidal gold particles. The gold-extract complexes were centrifuged at 1,500 rpm in a SW 40 rotor for 1 hour. The resulting pellet was discarded. The supernatant solution was centrifuged at 11,500 rpm (in the SW 40 rotor) for 1 hour and the supernatant solution was discarded. The pellet was suspended in 10 mL of PBS with 0.2 mg/mL PEG.

2.18 Wheat Germ Affinity Chromatography

Affinity chromatography of iodinated extract preparations was performed in glass wool-plugged pasteur pipets containing approximately 0.4 mL of wheat germ agglutinin-agarose (obtained from Pharmacia P-L Biochemicals Co., Dorval, Quebec), which had being thoroughly washed and equilibrated with 1% BSA in mQH_2O. The iodinated extract (200 uL) was diluted in column buffer (1% BSA in mQH_2O) and added to WGA-agarose. The suspension was mixed end over end for three hours at 4° C to allow binding to the affinity matrix. The WGA-agarose was then transferred to a glass wool-plugged Pasteur pipet. The unbound material was washed

out with 8 mL of 1% BSA in mQ H_2O and 0.5 mL fractions were collected. After the sixteenth fraction, bound glycoproteins were eluted with 8 mL of 0.5 M N-acetylglucosamine and another sixteen 0.5 mL fractions were collected. Aliquots of the peak fractions were analysed by SDS-PAGE.

3. RESULTS

3.1 Preliminary investigations

Initially the bacterial strains (H10407, CFA/I$^+$ and H10407P, CFA/I$^-$) were examined to confirm that they were identical in all respects except for the presence of CFA/I pili on H10407. Therefore, electron microscopy was performed on the bacterial strains after overnight growth (37° C) on CFA agar to: (1) confirm the presence of pili on the H10407 strain and the absence of pili on the H10407P strain; and (2) to evaluate the number of piliated organisms and the amount of piliation. Electron microscopy revealed that greater than 70% of the CFA/I$^+$ bacteria were multipiliated (Figure 1), whereas no pili could be seen on the CFA/I$^-$ bacteria. Electron microscopy revealed that both strains of *E. coli* possessed flagella.

The presence of CFA/I pili on the respective strains was also examined by MRHA of human erythrocytes, and bacterial agglutination using polyclonal and monoclonal antibodies directed against the CFA/I pili (Table3). Both procedures provided further evidence that the pili expressed by H10407 were indeed CFA/I pili and it confirmed the absence of this type of pilus on H10407P.

In addition, the two strains were examined for plasmid content (Figure 2) to ascertain the presence or absence of the 60 x 10^6 MW plasmid, which carries the genes encoding for CFA/I pili. The ethidium bromide stained agarose gel of plasmid DNA

FIGURE 1

Electron micrograph of piliated H10407 *E. coli* negatively stained with 1% PTA. Prepared as described in Material and medthods.

P = CFA/I pili; F = flagella

Bar = 1 micrometer

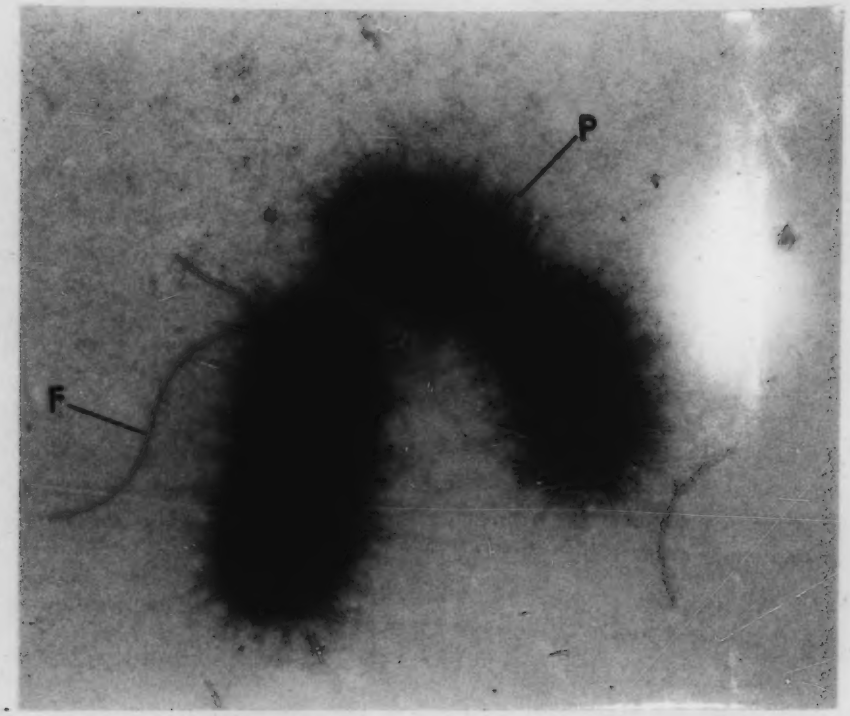

P
F

Table3

Agglutination Reaction of H10407 *E. coli* with Human and Guinea Pig Blood and Polyclonal Anti-CFA/I Pili Antibodies

	Human blood *	Guinea pig blood	Anti-CFA/I antibodies
H10407 (CFA/I^+)	(+)	(-)	(+)
H10407 (CFA/I^-)	(-)	(-)	(-)

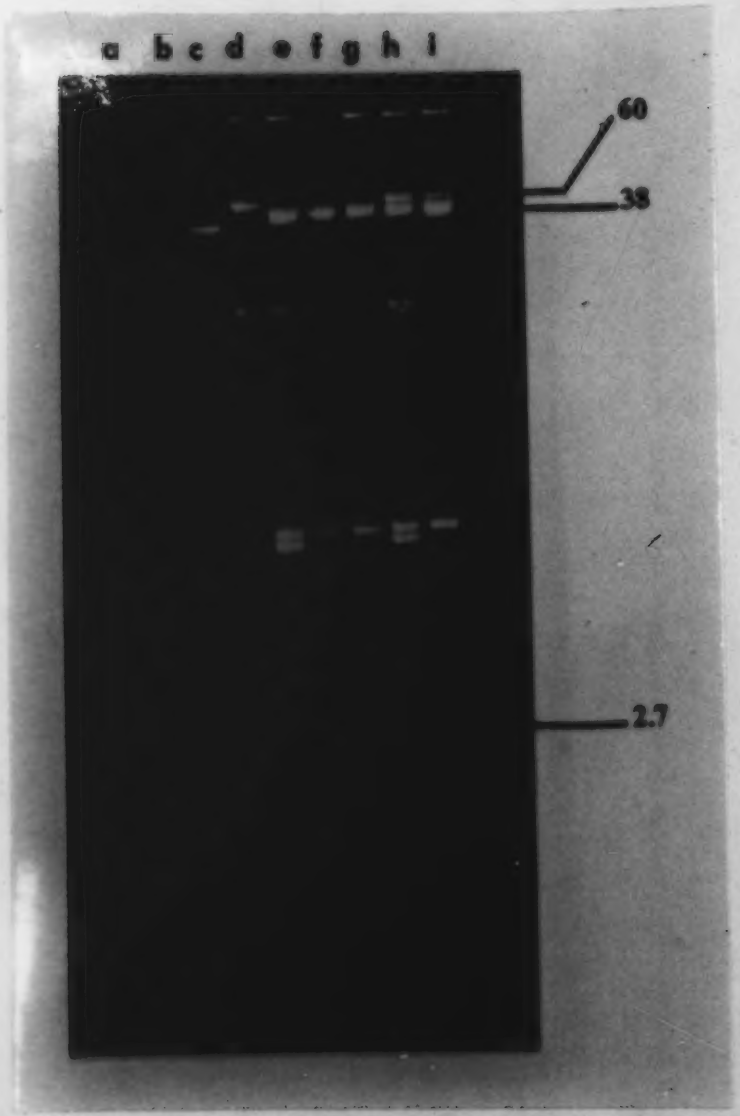
1) Washed erythrocytes (human or guinea pigs) were suspended in PBS (containing 1 mM Ca^{++} and 10 mM Mg^{++}) at a concentration of 10^8 cells/mL as determined by using a hemocytometer. Then 50 µL of blood was mixed with 50 µL of the respective bacterial strain (at a concentration of 3-5 x 10^6 cells/mL in PBS, 1 mMCa^{++} and 10 mMMg^{++}) on a glass slide and rocked for 1 minute at which time the hemaggluination reaction was noted.

2) 50 µL of each bacterial strain was mixed with 50 µL of polyclonal serum (anti-CFA/I pili, titre 1/32,000) on a glass slide, the mixtures were rocked for 1 minute and the agglutination reaction was recorded.

* PBS suspensions of human blood of groups A, B, and O were used both in the presence and absence of 0.2 M Mannose.

FIGURE 2

An ethidium bromide stained agarose gel of plasmid DNA obtained from enterotoxigenic *E. coli* isolates. Samples were analysed on 0.7% Tris borate (pH 8.8) agarose for 8.5 hours, at 100V (constant). Lanes a, b, c, and d contain plasmids of known molecular weights (2.7 Mdal, 23Mdal, 38 Mdal, and 60 Mdal respectively), used to calibrate the gel. Lanes e, f, and g contain the plasmids isolated from CFA/I$^-$ *E. coli* isolates; and lanes h and i contain the plasmid from CFA/I$^+$ *E. coli* isolates. The position of the molecular weight (Mdal) markers are shown on the right of the figure.


a b c d e f g h i
60
38
2.7

revealed that H10407 carried the 60 Mdal plasmid, which was found to be lacking in the H10407P strain.

Finally, LPS analysis of the H10407 and H10407P strains was performed. A silver stained SDS-PAGE revealed that both strains had similar LPS (Figure 3).

These preliminary investigations assured that any difference in the interactions of H10407 and H10407P was due to the presence of CFA/I pili.

3.2 Isolation and purification of CFA/I pili

CFA/I pili were purified by the procedure described in Materials and Methods 2.3. The quality of the purified pili preparation was determined by SDS-PAGE and electron microscopic examination. Pili preparations were considered to be pure when only one band could be detected in Coomassie blue stained SDS-PAGE gels and no flagella (the major contaminant) could be detected by either electron microscopy or SDS-PAGE.

On SDS-PAGE examination of at least 15 preparations, one major band was detected with an apparent molecular weight of 15,200 ± 800 (Figure 4). This value was in good agreement with that calculated from the published amino acid sequence for the CFA/I pilin subunit of 15,058 (57). Although the CFA/I$^+$ strain used in this study was judged to be free of type 1 pili by the absence of hemagglutination of guinea pig erythrocytes, the gel also indicated that the strains did not produce type 1 pili because

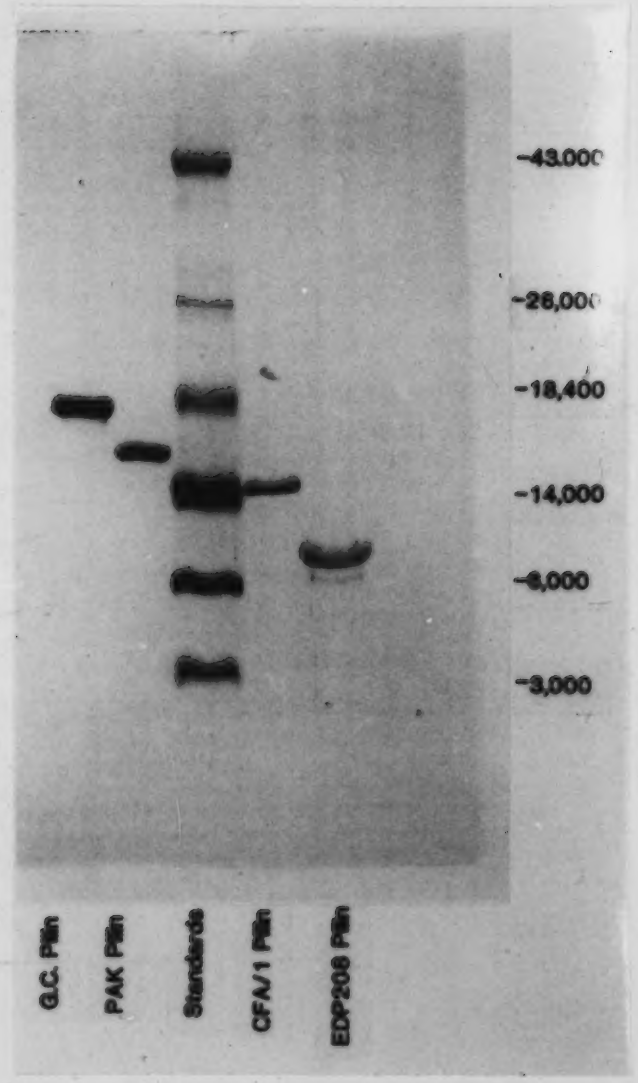
FIGURE 3

Silver stained SDS-PAGE to reveal the LPS moieties of the respective bacteria. Lane 1 contains molecular weight markers (their weight is indicated along the side). Lane 2 contains the LPS moities of H10407 (CFA/I$^+$) bacteria . Lane 3 contains the LPS of H10407P (CFA/I$^-$) bacteria .

1
2
3
92500
66200
45000
31000
21500
14400

FIGURE 4.

SDS-PAGE analysis of purified CFA/I pili. Purified CFA/I pili were subjected to discontinuous SDS-PAGE in the presence of β-mercaptoethanol using a 20% separating gel. The gels were stained with Coomassie blue and the positions of the standard proteins (low molecular weight mixture containing: ovalbumin - 45,000, α-chymotrypsinogen - 26,000, β-lactoglobin - 18,400, lysozyme - 14,000, bovine trypsin inhibitor - 6,000 and insulin A and B chains of 3,000 obtained from Bethesda Research Laboratories, Gaithersburg, MD) are indicated by their molecular weights on the right of the figure. Pili from *N. gonorrhoea* strain MS 11 (G. C. Pilin; molecular weight - 17,500, kindly provided by G. K. Schoolnik), *P. aeruginosa* strain K (PAK pilin; molecular weight - 15,000 and *E. coli* containing the plasmid pED208 (EDP208) pilin; molecular weight - 6,900 were analysed in addition to the CFA/I pili preparation to provide extra molecular weight standards.


-43,000
-26,000
-18,400
-14,000
-8,000
-3,000
G.C. Pilin
PAK Pilin
Standards
CFA/1 Pilin
EDP208 Pilin

no protein band with a molecular weight of 17,000 or aggregated material was seen in the numerous CFA/I pili preparations (80).

3.3 Bacteria binding assay

The initial objective was to investigate erythrocyte receptors using purified, [^{125}I]-labeled CFA/I pili. However, when iodinated probes are used for receptor studies it is imperative to demonstrate that the iodination procedure has not altered the binding characteristics of the iodinated ligand (3). This can be accomplished by demonstrating that the labeled ligand has retained its biological activity or that unlabeled ligand is capable of competing for receptor binding sites with the labeled probe. Since it was found that purified CFA/I pili do not cause hemagglutination, a characteristic which was reported previously (28), it was impossible to use the hemagglutination reaction to demonstrate retention of biological activity in iodinated CFA/I pili preparations. Therefore, we examined the integrity of the receptor binding site in iodinated CFA/I pili preparations by the competitive binding procedure.

However, when [^{125}I]-labeled CFA/I pili (>70% TCA precipitable counts) were used in erythrocyte competitive binding assays, we were unable to demonstrated any reduction in the number of bound [^{125}I] counts in the presence of excess unlabeled CFA/I pili. This suggested that: (1) iodination had altered the erythrocyte receptor binding site of CFA/I pili such that labeled and unlabeled pili were binding to erythrocyte membranes by

different mechanisms, or (2) the binding affinity for CFA/I receptors was so low that bound pili were being removed from the erythrocyte surface during the washing procedure required to separate erythrocyte-bound from unbound pili or, (3) the erythrocytes contained enough receptors to bind all of the labeled and unlabeled pili in the competitive binding assays. Although the last point (3) seemed unlikely because, in the presence or absence of unlabeled CFA/I pili, less than 1% of the input ^{125}I-CFA/I counts were bound to the erythrocytes, it was impossible to distinguish between the first two possibilities.

Since it was not possible to demonstrate that iodinated CFA/I pili retained the same binding characteristics as unlabeled pili, the decision was made to examine the interaction of whole bacteria with erythrocytes. The data from such experiments demonstrated that CFA/I$^+$ organisms bound significantly better to the erythrocyte membrane than CFA/I$^-$ organisms (Table4). Further, the addition of purified CFA/I pili to the reaction mixtures caused a reduction in the number of bound CFA/I piliated organisms to that of the level observed for the nonpiliated organisms (Table 4). These results suggested: (1) that the binding of CFA/I$^+$ organisms to the erythrocyte membrane is mediated by CFA/I pili; and (2) that there are at least two apparent mechanisms involved in the attachment of H10407 organisms to the erythrocyte surface. The first mechanism involves the specific interaction of CFA/I pili with its receptor(s) on the erythrocyte surface, and a second mechanism which is shared between CFA/I$^+$ or CFA/I$^-$ bacteria.

TABLE 4

Attachment of CFA/I+ and CFA/I- Bacteria to Erythrocytes (a)

H10407 Strain	Experimental Conditions	% Inocullum Bound ± S.D.	% Difference in Presence of Pili(b)
CFA/I+	(-)Pili(c),(+)RBC	22.9 ± 5.0	
CFA/I+	(+) Pili, (+) RBC	16.2 ± 3.0	-6.7 ± 8.0
CFA/I+	(-) Pili, (-) RBC	4.1 ± 2.7	
CFA/I-	(-) Pili, (+) RBC	12.0 ± 3.9	
CFA/I-	(+) Pili, (+) RBC	13.0 ± 4.9	+1.3 ± 8.8
CFA/I-	(-) Pili, (-) RBC	6.6 ± 2.0	

a) 3 - 5 x 10^6 colony forming units of H10407 CFA/I+ or CFA/I- bacteria (200 uL) were added to 4 x 10^7 erythrocytes (200 uL) and bacterial attachment was assayed as described in the text. The number of bound bacterial colony forming units was determined by plating 100 uL of the washed erythrocyte pellet on nutrient agar plates and the percent bacteria bound to the erythrocytes was calculated. Each value represents the mean of at least 6 determinations (± Standard deviation).

b) The difference in the attachment of piliated bacteria in the presence and absence of CFA/I pili was found to be statistically significant ($P<0.01$, as determined by the Student T Test). The difference in the attachment of nonpiliated bacteria in the presence and absence of CFA/I pili was found to be statistically insignificant ($P>0.2$).

c) Erythrocyte attachment experiments were performed in the presence or absence of 0.7 mg/mL purified CFA/I pili.

An electron microscopic investigation of the interaction between CFA/I$^+$ or CFA/I$^-$ bacteria and erythrocytes is shown in figure 5. The electromicrographs revealed that there was physical contact between CFA/I$^-$ bacteria, whereas there was always a gap seen in the interaction between CFA/I$^+$ bacteria and the erythrocyte's membrane; the CFA/I pili were not easily visible. When ruthenium red was used to examine the binding in greater detail, the pili on the CFA/I$^+$ organism became more visually prominent linking the organism to the erythrocyte membrane (Figure 5), whereas, with the CFA/I$^-$ organism, which was in physical contact with the erythrocyte's membrane revealed the presence of a dark staining material surrounding the bacterium (62).

Enzyme treated erythrocytes were also used in binding experiments with whole organisms. The results obtained from this investigation (Table 5) indicate that the receptor for CFA/I pili on the erythrocyte membrane is a sialoglycoprotein. This observation is consistent with previous findings reported in the literature (6).

3.4 Extraction of Human Erythrocyte Membranes with Lithium Diiodosalicylate

To investigate further the nature of the receptor a glycoprotein extract of erythrocyte membranes was prepared as described in section 2.7 of Materials and Methods. The extract (Figure 6) caused the (slide) agglutination of CFA/I$^+$ but not of CFA/I$^-$ organisms at a protein concentration of 0.5 mg/mL. This

FIGURE 5 Electron Microscopy

CFA/I$^+$ and CFA/I$^-$ bacteria were incubated with erythrocytes as described in Material and Methods. The erythrocytes were sedimented by centrifugation and the cell pellets were fixed at 4°C overnight in 3.5% glutaraldehyde (0.1M cacodylate buffer, pH 7.2) in the presence or absence of 0.075% ruthenium red. The fixed suspensions were washed three times in 0.1 M cacodylate buffer, pH 7.2. The specimens were post-fixed for 1 - 2 hours with 1.0% OsO_4 in cacodylate buffer. After washing to remove excess OsO_4, the cells were dehydrated with ethanol and propylene oxide, embedded in epon, sectioned, stained with lead citrate - uranyl acetate and examined in the electron microscope.

Electron micrographs of thin section of H10407 bacteria associated with human erythrocytes. Each photograph represents a 52,000X magnification. P = CFA/I pili; H = H10407 bacterium; E = erythrocyte.

(A) Ruthenium red-stained interaction of H10407 (CFA/I$^+$) bacteria with erythrocytes.

(B) Ruthenium red-stained interaction of H10407 (CFA/I$^-$) bacteria with erythrocytes.

(C) H10407 (CFA/I$^+$) bacteria interacting with erythrocytes, without ruthenium red stain.

(D) H10407 (CFA/I$^-$) bacteria interacting with erythrocytes without ruthenium red stain.

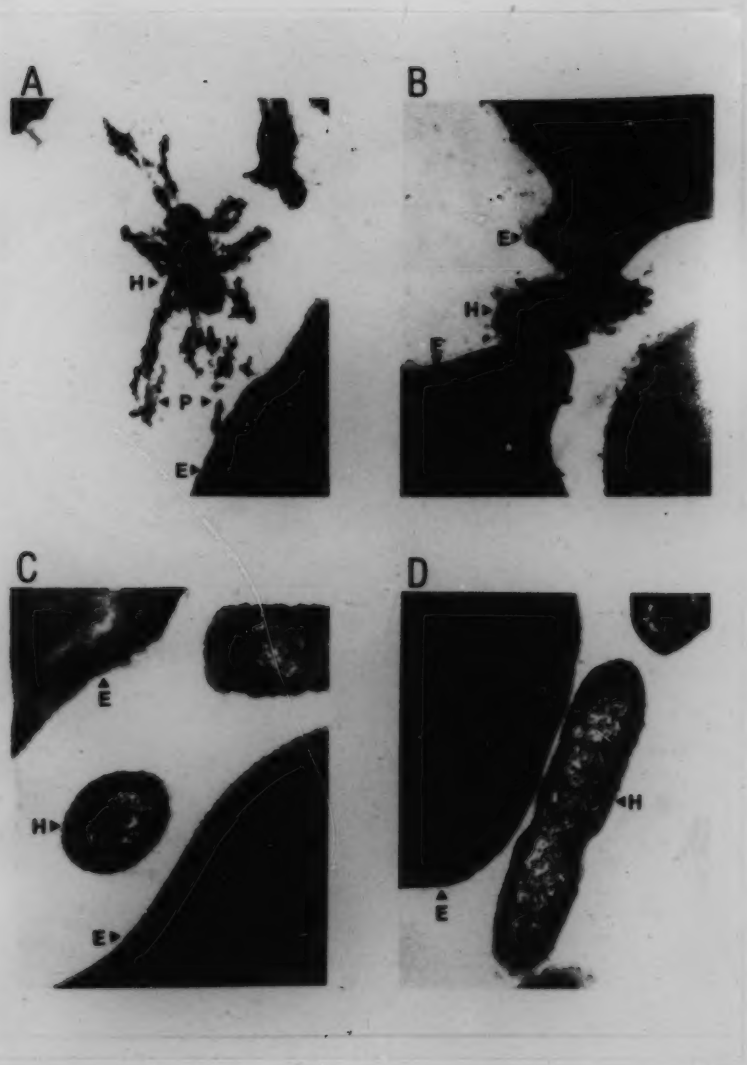

A
H
P
E
B
E
H
C
E
H
E
D
H
E

TABLE 5

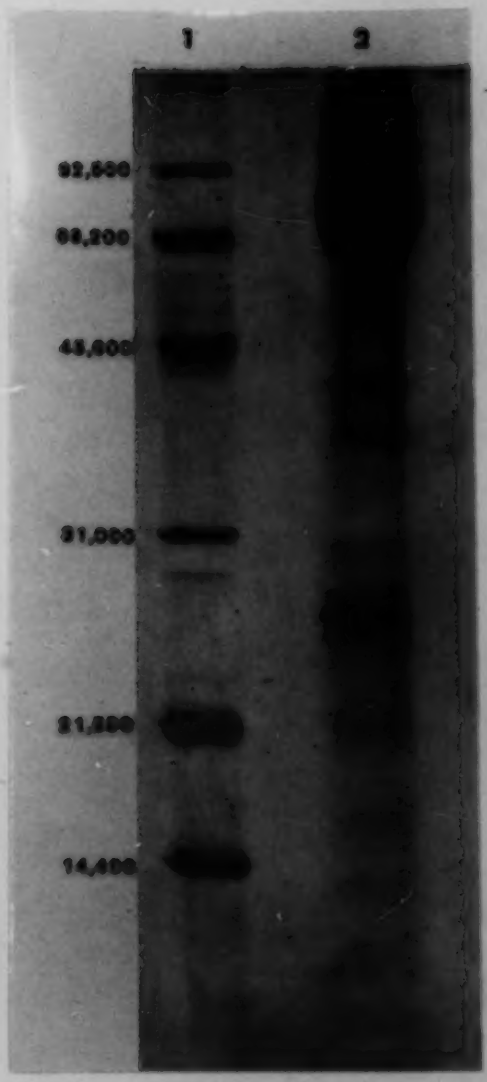
Attachment of H10407 Bacteria to Trypsin- or Neuraminidase-Treated Human Erythrocytes

Treatment	H10407 Strain	% Inoculum Bound ± S.D.
None	CFA/I+	33.7 ± 10.0
	CFA/I-	13.9 ± 4.2
Trypsin	CFA/I+	18.3 ± 4.3
	CFA/I-	15.1 ± 4.6
Neuraminidase	CFA/I+	19.4 ± 3.8
	CFA/I-	12.1 ± 1.0

3 - 5 x 10^6 colony forming units of H10407 CFA/I+ or CFA/I- bacteria (200 uL) were added to 4 x 10^7 erythrocytes (200 uL) and bacterial attachment was assayed as described in the text. The number of bound bacterial colony forming units was determined by plating 100 uL of the washed erythrocyte pellet on nutrient agar plates and the percent bacteria bound to the erythrocytes was calculated. Each value represents the mean of at least 6 determinations. The difference between the attachment of H10407 CFA/I+ bacteria to either trypsin- or neuraminidase-treated erythrocytes and untreated erythrocytes was found to be statistically significant ($p < 0.05$). The difference between the attachment of H10407 CFA/I- bacteria to either trypsin- or neuraminidase- treated erythrocytes and untreated erythrocytes was found to be statistically insignificant ($p > 0.2$).

FIGURE 6

SDS-PAGE analysis of "glycoprotein extract" from human erythrocyte membranes prepared by the lithium diiodosalicylate procedure as described by Marchesi & Andrews (1971). The extracts were subjected to discontinuous SDS-PAGE in the presence of β-mercaptoethanol using a 14% separating gel. The gel was silver stained. Lane 1 has molecular weight standards, lane 2 has glycoprotein extract .


1
2
92,500
66,200
45,000
31,000
21,500
14,400

suggested an interaction between components in the extract and CFA/I pili. Based on this observation a series of binding assays using extract and purified CFA/I pili, as well as a series of affinity isolation procedures using labeled extract and CFA/I+ and CFA/I- bacteria were performed.

The binding assays were done as described in section 2.11 of Materials and Methods. Binding assays using CFA/I pili-coated polystyrene test tubes and labeled extract were performed to establish the optimum temperature for the assay. The results (0° C= 2,732 ± 525 cpm; RT= 6605 ± 434 cpm, n=6) of this investigation indicated that room temperature (RT) was superior. Competition studies used to demonstrate specific binding of labeled extract to CFA/I pili also indicated that room temperature was the best temperature to perform the assay: at 0° C the net difference in binding was 1221 ± 924 cpm whereas, at RT the net difference was 4740 ± 703 cpm.

Binding assays (Table 6) also indicated that labeling the CFA/I pili with ^{125}I destroyed or altered its receptor binding domain, since unlabeled CFA/I pili did not compete for receptor binding sites with the labeled probe. However, [^{125}I]-labeling of the extract did not alter its receptor binding domain since unlabeled extract was able to compete for the CFA/I pili receptor site with the labeled extract.

Further experiments were performed to determine the minimum amount of purified pili that would be necessary for binding assays (Figure 7). The data indicate that 20 µg of purified CFA/I was sufficient for coating the polystyrene test tubes.

TABLE 6

Binding Assay

Polystyrene Test Tubes Coated with	Labeled Pili (μL)	Labeled Extract (μL)	Unlabeled Pili[1] (μL)	Unlabeled Extract[1] (μL)	CPM Bound[2] ±S.D.	Net Difference
CFA/I	--	10	--	--	4076 ± 995	2210 ± 1399
CFA/I	--	10	--	20	1866 ± 504	
Extract	10	--	--	--	3379 ± 1594	-292 ± 3249
Extract	10	--	20	--	3671 ± 1655	
BSA	10	--	--	--	1391 ± 70	
BSA	--	10	--	--	1013 ± 36	

Test tubes were coated with 50 μg of CFA/I or glycoprotein extract.

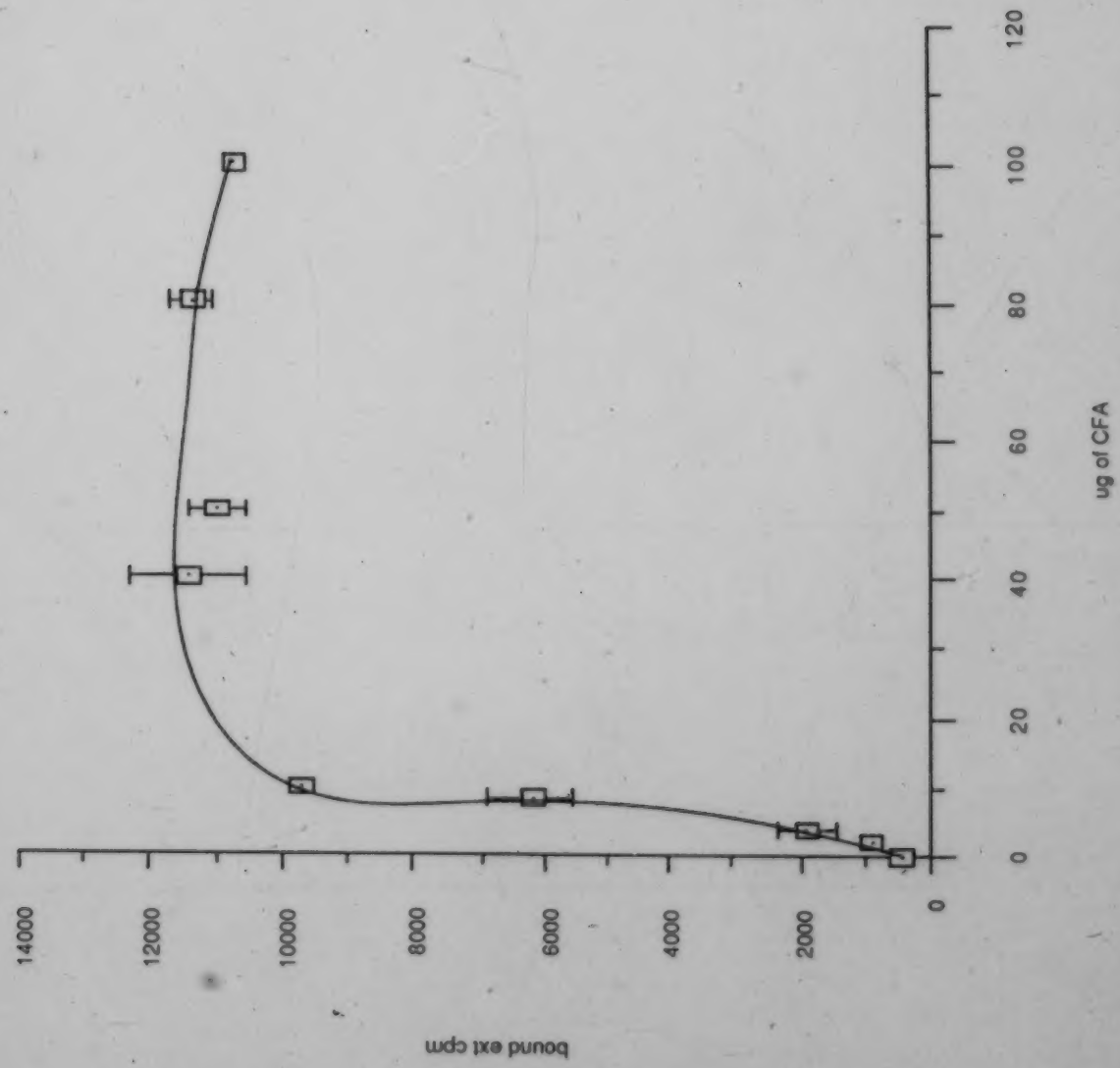
1) Glycoprotein extract concentration = 1.7 μg/μL (unlabeled)
CFA/I pili concentration = 1.0 μg/μL (unlabeled)

2) Results of two experiments performed in triplicate

3) The polystyrene test tubes were coated as described in Materials and Methods 2.11. The binding assay consisted of incubating the labeled component in the respective polystyrene test tubes for 3 hrs., in a total reaction mixture volume of 0.5mL mQ water (1% BSA). After 3 hrs., (at room temperature) these test tubes were washed 3 times with mQ water (1% BSA) and counted in a gamma counter.

FIGURE 7

Saturation binding experiment to establish the amount of pili required to saturate binding sites on the test tubes. The polystyrene tubes were activated with 2.5% glutaraldehyde and then increasing amounts of CFA/I pili were added. The tubes were washed and the remaining protein binding sites were blocked with 0.1% BSA. Binding of labeled extract was allowed to proceed for 3 hrs., at room temperature. The tubes were washed to remove unbound extract and the amount of bound radioactivity was determined in the gamma counter.


bound ext cpm
14000
12000
10000
8000
6000
4000
2000
0
0
20
40
60
80
100
120
ug of CFA

Finally, binding isotherm assays were performed to determine the physical characteristics of the extract-CFA/I receptor moiety interactions (Figure 8). Scatchard analysis of this data (Figure 8b) produced a straight line, indicating that: (1) there was no binding co-operativity, (2) the binding sites were homogeneous and (3) binding could be saturated. However, since the extract contained several components (Figure 6) one could not assign an affinity constant to the receptor.

The affinity isolation procedure (outlined in 2.12 of Materials and Methods) using labeled extract and CFA/I$^+$ and CFA/I$^-$ organisms revealed a 27,000 molecular weight band that was associated only with the CFA/I$^+$ organisms (Figure 9). Further,evidence for the specificity of the 27,000 molecular weight band for CFA/I pili was obtained by demonstrating that purified CFA/I pili were able to compete for binding with CFA/I$^+$ organisms (Figure 9, Lanes 4 & 5).

Although high molecular weight iodinated material which failed to enter the separating gels was also detected only in lanes containing CFA/I$^+$ bound extract components, it remains to be determined whether this represents aggregates of the 27,000 molecular weight CFA/I specific species or a unique erythrocyte receptor.

3.5 Erythrocyte versus *E. coli* H10407 Sensitivity to Glutaraldehyde Fixation

Erythrocytes diluted 1:10 in PBS, were fixed overnight at 4° C in increasing concentrations of glutaraldehyde (2.5 to 40%, also

FIGURE 8

Figure 8a. Binding isotherm data. Polystyrene tubes were activated and coated with 20 ug of CFA/I pili as described in Materials and Methods 2.11. Increasing amounts of [^{125}I]-labeled extract were then added to the tubes in the presence of 0.1% BSA and allowed to bind for three hours at room temperature. The tubes were washed and the amount of bound radioactivity was determined. The ug of bound and unbound extract was determined from the specific activity (protein concentration of the extract was determined by the Lowry procedure).

Figure 8b. Scatchard plot of equilibrium binding data of figure 8a

FIGURE 8a

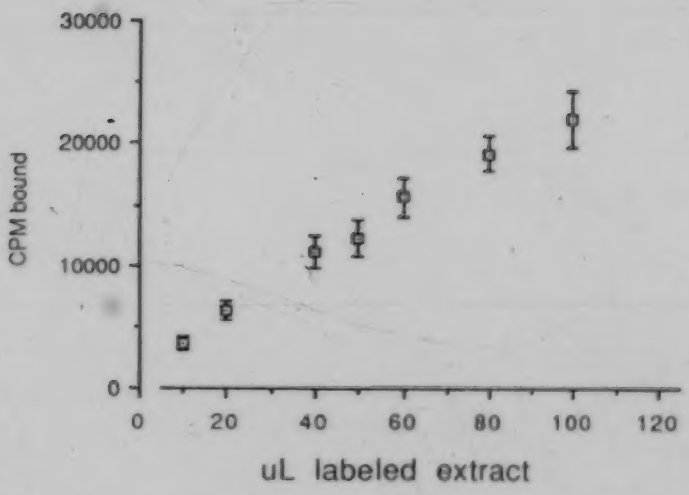


FIGURE 8b

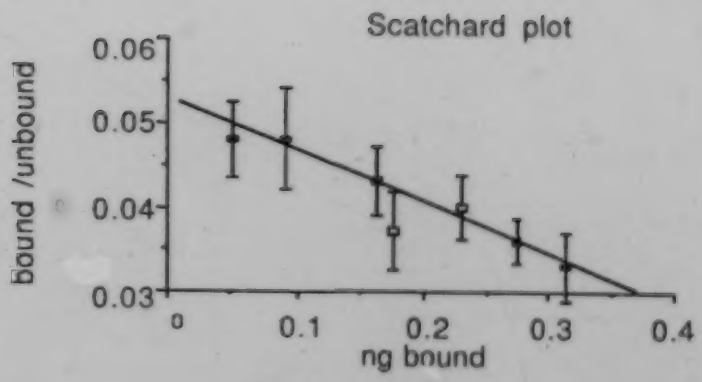
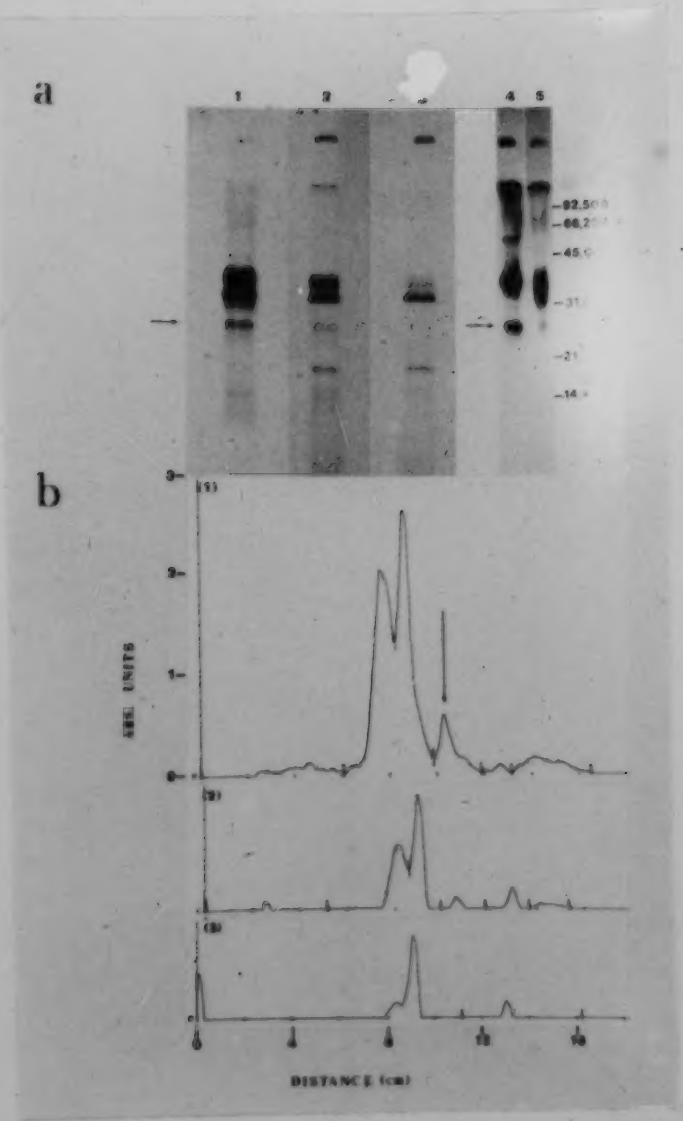


FIGURE 9.

Identification of human erythrocyte receptors for CFA/I pili. CFA/I$^+$ or CFA/I$^-$ organisms were incubated with [^{125}I]-labeled diiodosalicylate extracts of human erythrocyte membranes as described in Materials and Methods. After extensive washing to remove unbound material the organisms were subjected to discontinuous SDS-PAGE in the presence of β-mercaptoethanol using a 14% separating gel. Panel a. 48 h exposure autoradiogram of dried gel. Lane 1: total iodinated erythrocyte extract. Lane 2: extract components bound to CFA/I$^+$ bacteria. Lane 3: extract components bound to CFA/I$^-$ bacteria. Lane 4: (separate experiment) extract components which bound to CFA/I$^+$ bacteria in the absence of purified CFA/I pili. Lane 5: extract components which bound to CFA/I$^+$ bacteria in the presence of 1.5 mg/mL purified CFA/I pili. The positions of the molecular weight markers are shown on the right of the figure. Panel b. Densitometric scans of lanes 1, 2 and 3 in the original autoradiogram. The absorbance scale is the same for all three scans and the arrows indicate the 26,000 molecular weight band with preference for binding to CFA/I$^+$ organisms. This is a representative SDS-PAGE gel. The experiment was repeated three times.


a
1
2
4
5
b
(1)
(2)
(3)
DISTANCE (cm)

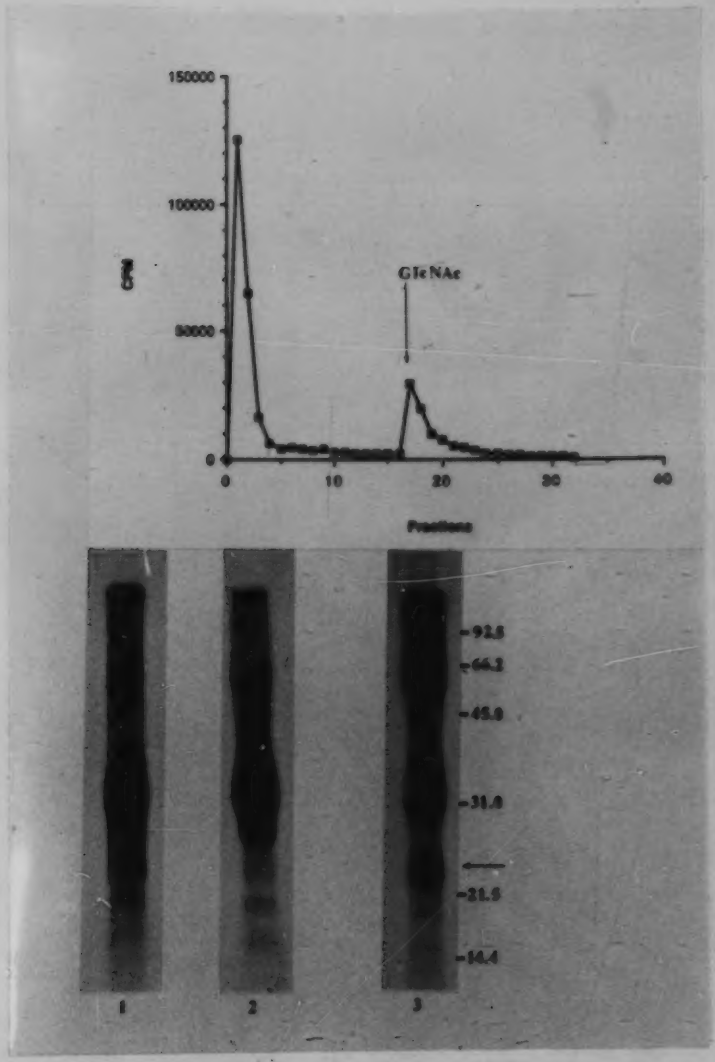
diluted with PBS). The following day, the erythrocytes were thoroughly washed in PBS and suspended to the same concentration in PBS containing 0.4M mannose. Glutaraldehyde-fixed red blood cells were agglutinated by the bacteria. The converse experiment was performed, whereby 400 µL aliquots of the bacteria (CFA/I$^+$) were fixed overnight in the same concentrations of glutaraldehyde (used for fixing the red blood cells). When the CFA/I$^+$ bacteria were mixed with erythrocytes (1/10 in PBS containing 0.4M mannose) hemagglutination was not observed. These results suggest that CFA/I pili are involved in the binding to the erythrocytes, since fixation with glutaraldehyde destroys the hemagglutination reaction. Whereas, the pili receptor on the erythrocyte's membrane was not sensitive to glutaraldehyde fixation therefore, its CFA/I pili binding domain is not a protein component (data not shown).

3.6 WGA Affinity Chromatography

Wheat germ agglutinin has affinity for N-acetyl β-D-glucosamine oligomers and N-acetyl β-D-glucosaminyl residues (84). To determine if the labeled extract contained such groups it was allowed to interact with WGA-agarose. After washing the mixture free of any unbound labeled extract, the bound material was eluted from the column with 0.5M N-acetylglucosamine. The data obtained indicates that the 27,000 MW band identified as the component responsible in binding to CFA/I pili possesses N-acetylglucosamine residues (Figure 10).

FIGURE 10.

Affinity chromatography of iodinated erythrocyte extract on wheat germ agglutinin (WGA)-agarose. In the upper portion of the figure 0.5 mL fractions were collected from the columns. Bound material was eluted (arrow) with 0.5 M N-acetylglucosamine (GlcNAc) in PBS. SDS-PAGE analysis and autoradiography of the column peak fractions is shown in the bottom portion of the figure. Lane 1: [^{125}I]-labeled extract. Lane 2: labeled extract components which failed to bind to WGA and appeared in fractions 1-5. Lane 3: labeled extract components which were eluted from the WGA with GlcNAc (fractions 17-20). The positions of the Coomassie blue stained molecular weight standards (molecular weights x 10^{-3}) are shown to the left of lane 3. The arrow beside lane 3 indicates the position of the 27,000 molecular weight CFA/I receptor species.


150000
100000
50000
0
0
10
20
30
40
GlcNAc
Position
-92.5
-66.2
-45.0
-31.0
-21.5
-14.4
1
2
3

3.7 *M. gallisepticum* Lectin Hemagglutination Inhibition Study

Based on the results obtained (Table 5) and the information available on the major components of the human erythrocyte membrane, it was suspected that glycophorin (MW 29,000) (18, 103) could be the receptor for CFA/I pili. To establish the role (if any) of glycophorin, a hemagglutination inhibition study using *M. gallisepticum* lectin and purified CFA/I pili was performed (Figure 11). The results show that purified CFA/I pili inhibited the binding of *M. gallisepticum* lectin to glycophorin, suggesting that glycophorin is involved in CFA/I pili binding.

3.8 Experiments designed to determine location of receptor binding domain of CFA/I pili

Five different monoclonal antibodies directed against the CFA/I pili (a gift from Dr. Paranchych) were obtained. It was of interest to see where they bound on the pilus (Figure 12) because these monoclonalsß inhibited hemagglutination. Therefore, it was assumed that these monoclonal antibodies either bound to the receptor binding domain on the pili or in some way sterically interfered with the binding to the erythrocyte membrane receptor. Protein A gold investigations were not elaborate enough to help elucidate the monoclonals' respective binding sites. Apparently, all monoclonals bound throughout the length of the pili and no distinct regions were observed for any of the monoclonal antibodies used. This same immunoelectron technique

FIGURE 11

M. gallisepticum lectin hemagglutination inhibition study. Wells in row A contain 2% erythrocytes in PBS , 0.4 M Mannose. Wells in row B contain CFA/I pili (0.5 mg/ml) and 2% erythrocytes (in PBS, 0.4M Mannose). Wells in row C contain *M. gallisepticum* lectin starting at 7.2 mg/ml then serially diluted two fold (left to right), and 2% erythrocytes (in PBS, 0.4M Mannose). Wells in row D contain pili (0.5mg/ml), 2% erythrocytes (in PBS, 0.4M Mannose), and lectin serially diluted left to right starting at 7.2mg/ml.

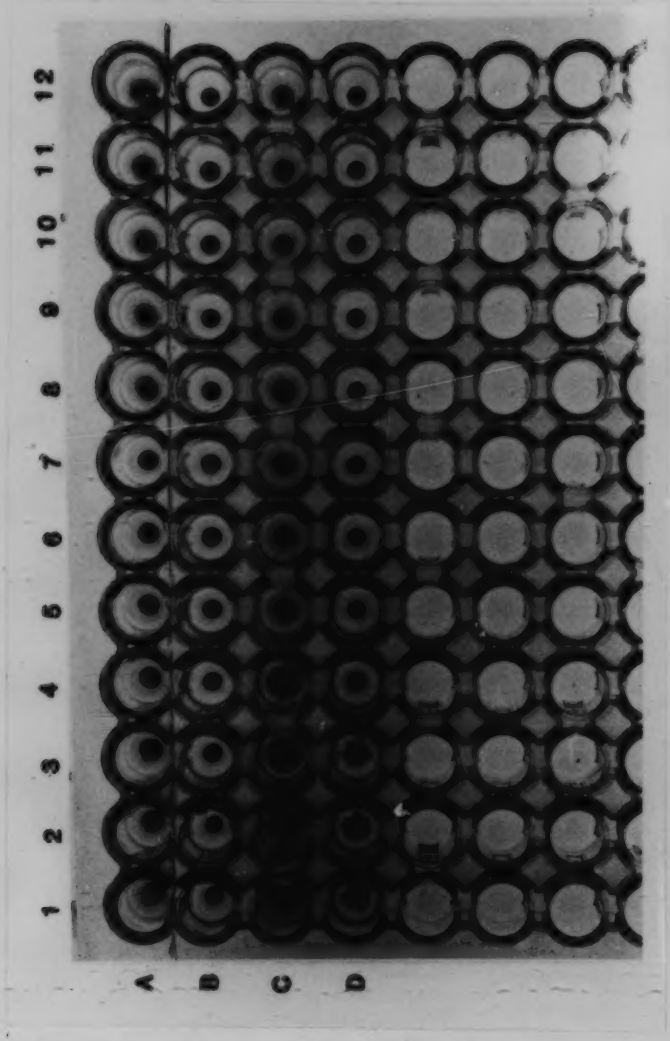

1 2 3 4 5 6 7 8 9 10 11 12
A B C D

FIGURE 12

Electron micrographs of Protein A gold studies using monoclonal antibodies, as described in materials and methods 2.16. The technique was used to visualize the various antigenic sites defined by the various monoclonal antibodies.

a) McAb #1

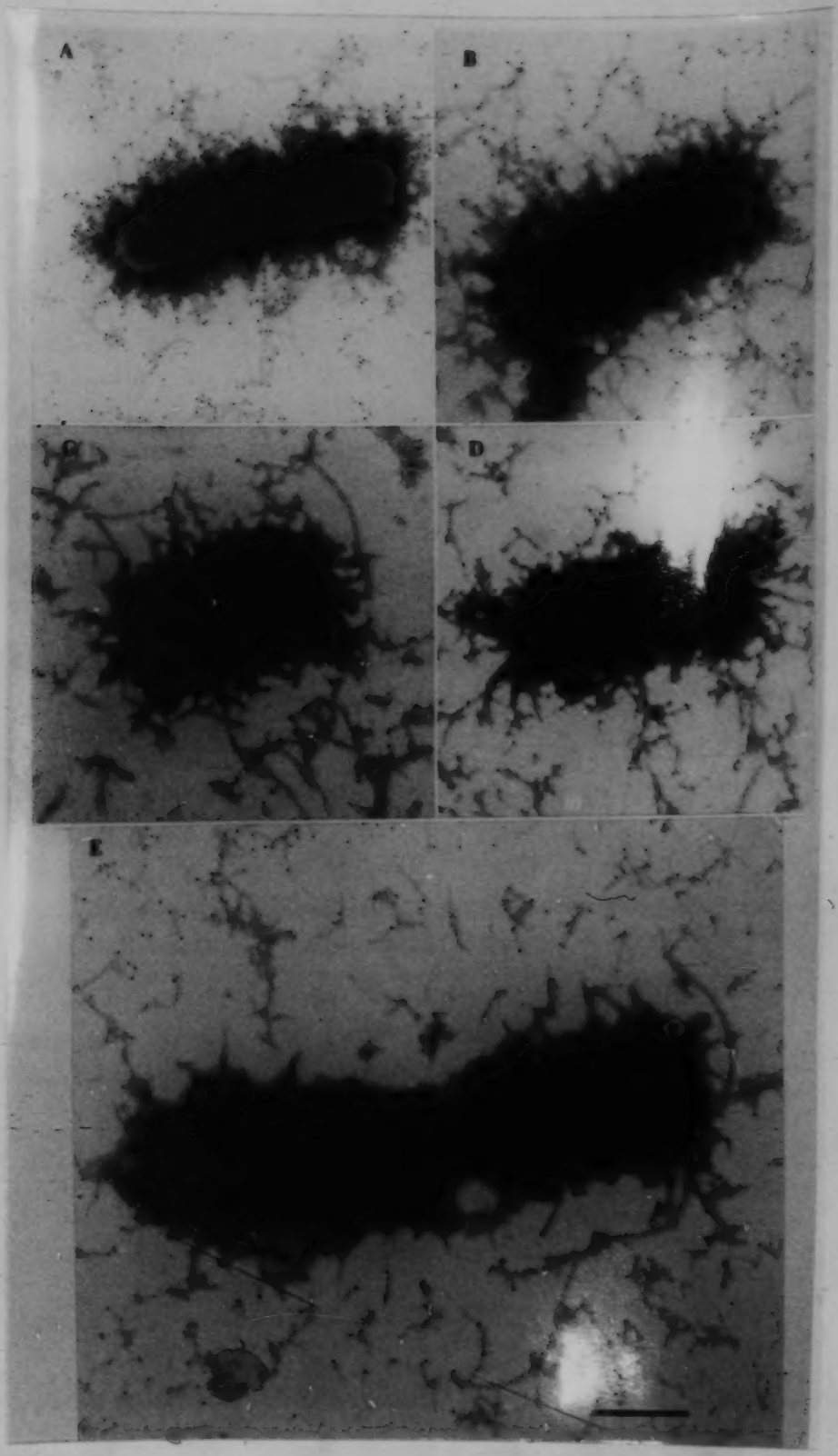
b) McAb #2

c) McAb #3

d) McAb #5

e) McAb #6

The magnification of each picture is 38,400X


A
B
C
D
E

had been used previously to locate an antigenic determinant of conjugative EDP208 F-like pili (118) to the pilus tip.

The conjugation of extract-colloidal gold was done in order to determine if the receptor binding domain was located at the pilus tip. By examining the extract-colloidal gold complexes with CFA/I$^+$ bacteria with the aid of an electron microscope it would have been possible to elucidate the region of the pilus tip involved in binding. Satisfactory and reproducible results were not obtained from this investigation; therefore, this approach was ended.

4. DISCUSSION

The objective of this investigation was to identify the human erythrocyte receptor for CFA/I pili. Therefore, the preliminary experiments were performed to confirm that the two strains of *E. coli* (H10407 and H10407P) were essentially identical aside from the presence of pili in H10407.

The presence or absence of the 60 x 10^6 MW plasmid encoding CFA/I pili was confirmed in the two strains of H10407 *E. coli* by the alkaline extraction of Birnboim and Doly (8). The expression of CFA/I pili was routinely determined by mannose resistant hemagglutination of human erythrocytes (MRHA), electron microscopy, and the agglutination of the CFA/I piliated organisms by poly-and monoclonal antibodies against the CFA/I pilus.

In addition, the presence of type 1 pili, which may be expressed by the two strains of H10407 *E. coli* were examined by mannose sensitive hemagglutination of guinea pig erythrocytes; type 1 pili were not detected by this procedure. Overnight culture (37° C) on CFA agar appears to suppress the expression of type 1 pili (31, 62). It was necessary to suppress type 1 pili expression in order to evaluate binding mediated by CFA/I pili.

The lipopolysaccharide (LPS) profiles of both strains were examined to ensure they were identical, since LPS may influence interactions between bacteria and erythrocyte membranes (11, 12, 93). The strains used in the investigation had a similar LPS

profile on examination by SDS-PAGE. Consequently, any influence mediated by the LPS moieties would be uniform for both strains.

After the preliminary investigations, the hemagglutination of human erythrocytes by CFA/I+ organisms was used as an assay to examine the interaction of the CFA/I pili and erythrocyte membrane receptor. From the binding studies a significantly higher amount of adherence to erythrocyte membranes was observed with piliated organisms compared to that of nonpiliated bacteria (Table 4). To determine the specificity of the binding for the CFA/I pili, purified CFA/I pili were used to compete for binding. The presence of purified CFA/I pili reduced the number of erythrocyte-bound piliated organisms to the level observed for the non-piliated organisms. This observation indicated that the binding of piliated organisms to the erythrocyte membrane was mediated by CFA/I pili (Table 4). A reason why so much purified pili were required to inhibit the binding of CFA/I+ organisms to erythrocytes can be based on: (1) the monovalency of the pili, based on electron microscopic observations (Figure 5); in addition to the physical observation that purified CFA/I pili does not cause hemagglutination and; (2) on the electron microscopic observation that CFA/I+ organisms were multipiliated therefore, therefore it would be easier for the organism to form a crosslink between erythrocytes than it would be to inhibit their binding.

When the binding of whole organisms to erythrocytes was examined (Table 4), there appeared to be two forms of attachment: one mediated by CFA/I pili and and the other which was unrelated to the presence of CFA/I pili and hemagglutination.

This observation can be interpreted in three ways: (1) the binding of CFA/I$^-$ organisms may have been an artifact resulting from the co-sedimentation of bacteria in the presence of erythrocytes during the centrifugation to remove unbound organisms; (2) CFA/I$^-$ binding may have been the result of hydrophobic or ionic interactions between CFA/I$^-$ bacteria and the membrane components which were also observed to bind to both CFA/I$^+$ and CFA/I$^-$ bacteria in the affinity isolation procedure (Figure 8); or (3) the presence of type 1 pili on both strains of bacteria. The co-sedimentation theory is the most likely explanation for the apparent binding of CFA/I$^-$ organisms to erythrocytes because the effect of trypsin on human erythrocytes is to remove the extracellular domains of glycophorin, the major sialoglycoconjugate and contributor of negative charge to the surface of the erythrocyte membrane (77, 104, 105, 107). The removal of sialic acid groups by neuraminidase treatment would also result in a decrease in the surface negativity of erythrocytes. If the CFA/I$^-$ organisms were binding to erythrocytes through ionic or hydrophobic mechanisms then the alteration in erythrocyte surface charge by the trypsin or neuraminidase treatments should have resulted in a change in CFA/I$^-$ binding to erythrocytes and this was not observed (Table 5). The removal of mannose-containing glycoproteins should also have resulted in a decrease in attachment if binding were due to type 1 pili, an unlikely event in any case because the growth of H10407 bacteria on CFA agar suppresses the production of their type 1 pili (31, 62).

To elucidate further the nature of the erythrocyte receptor, binding experiments with trypsin- or neuraminidase-treated erythrocytes were performed. The data in table 5 demonstrated that the CFA/I pili receptor on the erythrocyte membrane may be a sialoglycoprotein, due to its sensitivity to these enzymes. The major sialoglycoprotein present on the erythrocyte membrane which is sensitive to both enzymes is glycophorin (77, 103, 104). Therefore, to evaluate the possible role of glycophorin as the erythrocyte receptor for CFA/I pili, an experiment was performed using a glycophorin-specific lectin isolated from *Mycoplasma gallisepticum* (4, 46), which hemagglutinates human erythrocytes. The assay was designed to demonstrate whether purified CFA/I pili could inhibit the *M. gallisepticum* lectin mediated hemagglutination reaction. The decrease in the hemagglutination titer of *M. gallisepticum* lectin in the presence of purified CFA/I (Figure12), suggested that the CFA/I receptor is either: (1) glycophorin, or (2) in the same plane as glycophorin such that the binding of CFA/I pili to their receptor(s) sterically hinders the *M. gallisepticum* lectin from binding to glycophorin.

To identify receptors directly, an extraction procedure was performed for the isolation of glycoproteins from the erythrocyte membranes. An extract was obtained, which caused the agglutination of piliated but not of non-piliated organisms. This suggested an interaction between extract components and CFA/I pili. Similar results were obtained by Bartus et al. (6). They isolated a sialoglycoprotein fraction (using the lithium diiodosalicylate procedure) from the erythrocyte membrane

which inhibited MRHA of CFA/I piliated organisms. They also reported that this sialoglycoprotein fraction inhibited MRHA eight times more effectively than did free sialic acid.

The isolation of a water soluble glycoprotein extract allowed investigation of ionic charge interference in interactions between extract and CFA/I piliated bacteria. This preliminary study suggests that ionic interactions do influence the association between CFA/I and its erythrocyte sialoglycoprotein receptor(s) present in the extract, since by increasing the ionic strength of the mixture it prevented the agglutination of piliated bacteria. This question could not have been answered using intact erythrocytes, since they are sensitive to osmotic variability.

The extract and purified CFA/I pili were also used to investigate the physical characteristics of CFA/I-erythrocyte receptor interactions. This was accomplished by performing binding experiments using extract and CFA/I pili coated polystyrene test tubes. The binding of [^{125}I]-labeled CFA/I pili to extract-coated polystyrene test tubes and that of [^{125}I]-labeled extract to CFA/I-coated test tubes (Table 6) was examined. When the experiments were performed with [^{125}I]-labeled CFA/I pili, very little binding was observed and unlabeled purified CFA/I pili did not compete for binding. However, when the experiments were performed with [^{125}I]-labeled extract, significant binding was observed and was reduced in the presence of unlabeled extract. These results suggested that the [^{125}I] radio-iodination procedure (which labels tyrosine residues), destroyed the binding activity of CFA/I pili but had no effect on extract receptor activity.

This observation suggested that tyrosine residue(s) in the CFA/I pili play a role in the binding to the erythrocyte extract. When these residues are iodinated, the structural integrity of the binding domain is altered or destroyed. The receptor binding domain of CFA/I pili may be similar to that of pertussis toxin which has been reported to be sensitive to damage by the same iodination procedure (3). Based on these observations, all subsequent experiments were performed using [^{125}I]-labeled extract bound to CFA/I-coated polystyrene test tubes.

When the binding data were analysed by the Scatchard procedure a straight line was obtained. This indicated that: (1) there was no binding co-operativity, (2) binding sites were homogenous, and (3) binding can be saturated. Extrapolation of the graph suggested that 20 μg of pili bind approximately 0.37 ng of extract. However, this is not certain, since it is not known how much pili remained bound to the polystyrene test tubes.

Once the physical properties of the erythrocyte receptor were documented, the task was to identify the component(s) in the extract. This was accomplished by incubating the CFA/I$^+$ and CFA/I$^-$ organisms with aliquots of [^{125}I]-labeled extract and identify the component(s) that bound to each by electrophoretic separation on SDS-polyacrylamide gels. Autoradiograms of the gels revealed dissimilarities in binding properties between the two strains.

Indeed, autoradiograms revealed: (1) several components of the labeled extract bound to both strains of bacteria, and (2) the

presence of a 27,000 ± 1,500 molecular weight component was seen in the CFA/I+ piliated bacteria (Figure 9) preparations.

Densitometric analysis (figure 9 Panel b) of the autoradiograms confirmed the presence of the 27,000 molecular weight band with piliated organisms. This molecular weight band corresponds approximately to the molecular weight of glycophorin A in SDS-polyacrylamide gels (18, 42). Additional evidence for the specificity of the 27,000 molecular weight band for CFA/I pili was obtained by demonstrating that purified CFA/I pili was able to compete for its binding to CFA/I+ organisms (Figure 9 panel A).

The data in figure 9 (panels A and B), and Table 4 also suggest that non-specific surface properties, such as LPS and glycocalyxes may be involved in the adherence of H10407P *E. coli* to human erythrocyte membranes.

Collectively, the data obtained, along with information reported regarding erythrocyte membrane components, suggested that the erythrocyte membrane receptor for CFA/I pili is glycophorin A, with the important moieties being the oligosaccharide chains. Overnight glutaraldehyde fixation of erythrocytes at 4° C, prior to performing a hemagglutination with the respective bacteria, did not alter hemagglutination, since glutaraldehyde does not fix sugars as it does proteins. In addition, the removal of sialic acid (Table 5), commonly found on membrane oligosaccharide chains, significantly reduced the binding of piliated organisms.

Further support for the identification of the CFA/I pili receptor on the human erythrocyte membrane could be obtained

by performing: (1) binding assays with En(a-) human erythrocytes, which lack glycophorin (107); (2) hemagglutination reaction evaluation after sequential treatment of erythrocyte membranes with glycosidases and (3) by electroeluting the 27,000 molecular weight band from the SDS-polyacrylamide gel and raising polyclonal antibodies to it. These antibodies could then be used to see if they inhibited hemagglutination of CFA/I+ organisms. Finally, the antibodies could be used to isolate the receptor from the erythrocyte membrane extract. Once the receptor component is isolated and purified, its interaction with CFA/I pili may be characterized by amino acid/sugar analyses and more complete kinetic studies.

It would be of interest to establish conclusively that glycophorin A is the erythrocyte receptor for CFA/I pili, for the two different sugar chains on the molecule are similar to residues found on many other glycoproteins. If these sugars are essential structural components of the receptor, the data could be extrapolated to other glycoproteins, such as those found on epithelial cells on the intestinal mucosa. It may then be reasonable to consider vaccine development, based on: (1) synthetic peptides representing CFA/I pilus receptor binding domains, or (2) novel pharmacological compounds designed to block pilus-receptor interactions in acutely symptomatic patients.

Electron microscopy (Figure 5) revealed that the pili bound to intact erythrocyte membranes at the distal end. This information is in agreement with that obtained in the study of Knutton et. al. (62). The monovalency of the CFA/I pilus may

explain why purified pili do not cause hemagglutination of human erythrocytes - they are not able to crosslink erythrocytes, unlike the multipiliated CFA/I$^+$ bacteria. It also raises the following question: does the pilus tip have a specific binding adhesin separate from the structural pilin component, or does the binding site consist of a three dimensional configuration of the terminal pilin subunit? Within the last two years, it has been observed that the adhesive properties of type 1 and P pili reside in a protein different from the pilin subunits (72, 78, 79). Based on these reports, it was hypothesized that such a system may exist for CFA/I pili. Therefore, it is possible that one or more of the minor bands often seen in SDS-PAGE preparations of purified CFA/I pili is the adhesin responsible for mediating MRHA and binding to human intestinal epithelial cells.

Conversely, these bands may represent contamination resulting from the purification procedure. If this is the case, the adhesive property of CFA/I pili is conferred by its sequence and three dimensional structure, like gonococcal pili (96). This structure (whether it is a separate adhesive component or the pili themselves) could conceivably recognize many related sialoglycoconjugates (such as those of erythrocytes and intestinal mucosa epithelia), with differing affinities.

Electron microscopic investigations were also performed to determine the location of the receptor binding site(s) of CFA/I pili. Colloidal gold (10-15 nm) particles were coupled to the erythrocyte membrane extract, presumably to distinguish specific regions of the pilus tip involved in adherence. Unfortunately, this

experiment failed to produce convincing and reproducible results. The coupling efficiency between the colloidal gold and extract components was questioned. It was performed at an acid pH, reported to be a requirement for optimum glycoprotein-colloidal gold conjugation (44). However, the coupling of gold particles to protein is best performed at the protein's isoelectric point. This extract had several components and it may have been too heterogeneous to be used in colloidal gold experiments of this nature.

Protein A gold (*Staphylococcus aureus* protein A linked to colloidal gold particles) studies were also performed to localize ultrastructurally the various antigenic sites against which several monoclonal antibodies (McAb) were directed. Electron microscopy revealed that these monoclonals bound to externally-exposed lateral sites on the CFA/I pili (figure 12).

In conclusion the results of this investigation have supported the earlier finding of Bartus et al. (6) that the human erythrocyte receptor for CFA/I pili is a sialoglycoprotein due to its sensitivity to neuraminidase and trypsin treatment. As well, the procedures used in this study have allowed the identification of the CFA/I pili erythrocyte receptor, a 27,000 MW moiety from a mixture of glycoproteins extracted from erythrocyte membranes.

The identification of this receptor has ultimately laid the grounds from which one can proceed to isolate, purify, and fully characterize the erythrocyte receptor for CFA/I pili. Then one can compare the erythrocyte sialoglycoprotein and its actual binding site structure to the sialoglycoproteins found on the intestinal

mucosa. Once this is done one can answer the question if the CFA/I pilus contains two independent receptor binding domains on one or more adhesins, each strictly recognizing different receptors or one binding sited which is flexible enough to recogmize more than one receptor? And this will eventually aid in the design of highly effctive inhibitors for the prevention of colonization of ETEC expressing CFA/I pili.

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Appendix: 1

- CFA plates:
  - 7.5 g of casamino acids
  - 1.13 g of yeast extract
  - 0.750 mL of 5% $MgSO_4$ (stock sol.)
  - 0.075 mL of 5% $MnCl_2$ (stock sol.)
  - 15.0 g of agar
  - 750 mL of dd H_2O
  - pH to 7.4 (approx)
  - autoclave and dispense

- TSA plates:
  - use tryptose blood agar base with yeat extract (dehydrated) final pH 7.3.
  - autoclave and dispense

- L broth:
  - 10 g Tryptone
  - 5 g yeast extract
  - 10 g NaCl
  - 1000 mL dd H_2O
  - pH 7.2
  - autoclave and dispense

Appendix: 2

Laemmli gels

12.5% Separating Gel	
Acrylamide; BIS (30:0.8)]	12.5 mL
1.875 M Tris, pH 8.8	6.0 mL
0.2 M disodium EDTA	0.3 mL
Milli-Q™ water	10.9 mL
TEMED (add before persulfate)	0.015 mL
10% Ammonium persulfate (fresh)	0.3 mL
	30.015 mL

5% Spacer Gel	
Acrylamide:BIS (30:0.8)	2.5 mL
1.0 M Tris, pH 6.8	1.88 mL
0.2 M disodium EDTA	0.15 mL
Milli-Q™ water	10.3 mL
TEMED (add before persulfate)	0.008 mL
10% Ammonium persulfate	0.15 mL
	15.0 mL

Laemmli Running Buffer (in 5 liters Milli-Q™ Water

Tris (Sigma 7-9)	15.125 g
Glycine	72.0 g
SDS (BDH of Bio-Rad *only*)	5.0 g

Laemmli sample buffer (LDM)

10% SDS (BDH)	2.0 mL
Dithiothreitol (DTT)	0.078 g (=50mM final)
-or- (*B* - mercaptoethanol)	(0.4 mL)
glycerol (glycerin)	1.0 mL
1.0 M Tris pH 6.8	0.625 mL
Milli -Q™ Water	6.0 mL
Saturated bromphenol blue (in water)	0.075 mL
	10.025 mL

Appendix: 3

BUFFER SOLUTION FOR THE DIRECT ELISA
SOLUTIONS

Coating Buffer

1.59 g. Na_2CO_3
2.93 g. $NaHCO_3$
0.2 g. NaN_3
1000 ml. H_2O
pH = 9.6

Phosphate-buffered saline Tween Buffer (PBS.T)

8.0 g. NaCl
0.2 g. KH_2PO_4. 12 H_2O (0.46 g. without H_2O; 0.75 g. with 7 H_2O)
0.2 g. KCl
0.5 mL Tween 20
1000 mL H2O pH = 7.4

PBST.BSA

TO PBS.T add 1% BSA w/v

10% Diethanolamine Buffer
97 mL diethanolamine
800 mL H_2O
0.2 g NaN_3
Adjust pH to 9.8 with conc. HCl then make up to 1000 ml.

Appendix: 4

BUFFERS FOR THE BIRNBOIM-DOLY PLASMID EXTRACTION PROCEDURE

Solution 1 (1 mL)

0.025 mL Tris (1 M, pH 8)
0.005 mL 40% glucose
0.95 mL H_2O
0.04 mL EDTA (0.25 M, pH 8)

- mix in a bulk solution and add below components when ready to use solution 1.

0.01 mL 5 mg/mL RNase
0.002 g lysozyme

Solution 2

0.2 mL NaOH (10 N)
0.5 mL 20% SDS
9.3 mL H_2O

- use within a week of making up solution

Solution 3

3 M NaOAc pH 4.8

Appendix: 5

Lowry Assay

1. Solutions

Solution A:

- 4 g NaOH
- 20 g Na_2CO_3
- 0.2 g KNa Tartrate
- Dilute to 1L with dd H_2O

Solution B:

- 0.2 g $CuSO_4 \cdot 5H_2O$

Solution C:

- 1 part Solution B:50 parts Solution A

2. Procedure

- use 0.1% BSA (PBS) as the standard protein
- add 10-60µg of your standard protein to respective test tubes with a final volume of 200 µL.
- add to these test tubes 400 µL of solution C
- incubate for ten minutes at room temperature
- the add 50 µL of Folin Reagent (diluted 1:1 in PBS)
- incubated for 30 minutes at room temperature
- then read absorbance at 600nm

APPENDIX 6

PHOSPHATE BUFFER SOLUTION

To make 10X PBS:

KCl	2g
KH_2PO_4	2g
NaCl	80g
$Na_2HPO_4\,7H_2O$	21.6g

add ddH_2O to 1 L mark and autoclave

to make 1 L of PBS with Mg^{+2} and Ca^{+2}
add 1 mL of 0.1M $CaCl_2$ and 10 mL of 01M $MgSO_4$ which have been autoclaved separately before adding it the PBS .

APPENDIX 7

CESCIUM CHLORIDE GRADIENT

layer the following CsCl gradients on top of each other:

5 mL-at a density of 1.50040
4 mL-at a density of 1.40000
2.5 mL-at a density of 1.30057
2.5 mL-at a density of 1.2006
2.5 mL-at a density of 1.10075

then add the pili prep solution to this gradient.